



Dipartimento di Ingegneria per l'Ambiente e il Territorio e Ingegneria
Chimica
Scienze ed Ingegneria dell'Ambiente, delle Costruzioni e dell'
Energia (SIACE)

CHIM/07 FONDAMENTI CHIMICI DELLE TECNOLOGIE
Ciclo XXIX

PhD Thesis

**PERFORMANCE OF HOLLOW FIBER MEMBRANE
BIOREACTOR AS A BIOARTIFICIAL LIVER**

PhD Student

Haysam Mohamed Magdy Ahmed

Supervisors

Dott.sa. Loredana De Bartolo
Prof. Erem Curcio

Coordinator: Prof. Pietro Pantano

Host Institute: Istituto per la Tecnologia delle Membrane
Consiglio Nazionale delle Ricerche (ITM-CNR)

February 22, 2017

Current research was funded by:



Marie Skłodowska-Curie Actions
Initial Training Network
Project BIOART
(Grant no.316690, EU-FP7-PEOPLE-ITN-2012)

Contents

Acknowledgement	6
Abstract (Italian)	7
Abstract	10
Preface and Scope of Work.....	12
CHAPTER 1	14
State of the art and research scope.....	14
1.1 Liver anatomy and physiology.....	14
1.2 Cellular components of the human liver	17
1.2.1 Hepatocytes	18
1.2.2 Sinusoidal endothelial cells	18
1.2.3 Stellate cells	19
1.2.4 Kupffer cells.....	19
1.2.5 Cholangiocytes.....	20
1.3 Liver support systems	20
1.3.1 Artificial liver support systems	20
1.3.2 Bioartificial liver support systems	22
1.3.3 Hepatocyte transplantation.....	27
1.3.4 Repopulation of decellularized liver	28
1.3.5 Organ printing.....	28
1.3.6 Induced organogenesis	29
1.4 Cell source as critical issue for BAL devices.....	30
1.4.1 Primary hepatocytes.....	30
1.4.2 Cell lines.....	31
1.4.3 Stem cells.....	31
1.5 Oxygen supply	33
1.7 Bioreactor configurations in bioartificial liver designs.....	35
1.5.1 Bioartificial liver systems in clinical trial	35
1.5.2 Bioreactor designs for in vitro hepatic culture studies	38
CHAPTER 2	41
Long-term Maintenance of Human Hepatocyte Microtissue Spheroids in a crossed hollow fiber membrane bioreactor	41
2.1 Introduction.....	41
2.2 Materials and methods	44

2.2.1 Mold fabrication	44
2.2.2 Bioreactor	44
2.2.3 Cell Culture	46
2.2.4 Cell Morphology	47
2.2.5 Assay of metabolic functions	48
2.2.6 Dissolved oxygen concentration measurement	49
2.3 Results	50
2.3.1 Morphological evaluation.....	50
2.3.2 Hepatocyte-specific functions.....	56
2.3.3 Oxygen uptake	59
2.4 Discussion.....	60
2.5 Conclusion	62
CHAPTER 3	63
3D organotypic liver organoid formation in hollow fiber membrane systems .	63
3.1 Introduction.....	63
3.2. Materials and Methods.....	65
3.2.1. Membrane systems	65
3.2.2. Cell Culture.....	66
3.2.3 Cell Morphology	68
3.2.4 Assay of metabolic functions	69
3.2.5. Statistical analysis	70
3.3. Results.....	70
3.3.1 Morphological evaluation of organotypic membrane system	70
3.3.2 Hepatocyte-specific functions.....	71
3.3.3 Oxygen uptake rate	80
3.4. Discussion.....	82
3.5. Conclusions	85
CHAPTER 4	86
Expansion and differentiation of stem cells in the hollow fiber membrane bioreactor	86
4.1 Introduction	86
4.2 Materials and Methods.....	88
4.2.1 Hollow-fiber membrane bioreactor	88
4.2.2 Cell Culture.....	88
4.2.3 Hepatogenic Differentiation	88

4.2.4 Cell Morphology	89
4.2.5 Assay of metabolic functions	90
4.2.6 Periodic Acid-Schiff (PAS) Stain for Glycogen	90
4.2.7 Total RNA Isolation and Reverse-Transcription Polymerase Chain Reaction	90
4.2.8 Statistical analysis	91
4.3 Results	92
4.3.1 Morphological evaluation of differentiating cells on various coatings	92
4.3.2 Gene expression profile of treated cells	95
4.3.3 Hepatocyte specific functions	96
4.4 Discussion.....	98
4.5 Conclusions	99
General Conclusion and Future Perspectives	101
References	104

Acknowledgement

Firstly, I would like to express my sincere gratitude to my advisor Dr. Loredana De Bartolo for the continuous support throughout my PhD study, for her patience, motivation, and immense knowledge. Her guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my PhD study.

Besides my advisor, I would like to thank Dr Efrem Curcio and Dr. Lidietta Giorno for their insightful comments and encouragement.

My sincere thanks also go to Dr. Simona Salerno, Dr. Antonella Piscioneri, and Dr. Sabrina Morelli, who provided me an opportunity to join their team, and who gave access to the laboratory and research facilities. Without their precious support it would not be possible to conduct this research.

I thank my fellow labmates in for the stimulating discussions, for the sleepless nights we were working together before deadlines, and for all the fun we have had in the last three years.

Last but not least, I would like to thank my family: My wife, parents and brother for supporting me spiritually throughout writing this thesis and my life in general.

Abstract (Italian)

C'è una crescente necessità di sviluppare un dispositivo bioartificiale di tipo epatico da utilizzare sia in applicazioni in vitro, per la sperimentazione della tossicità di molecole da parte delle aziende farmaceutiche, e sia in applicazioni cliniche per supportare pazienti con insufficienza epatica in attesa di trapianto di organo. A tale scopo è stato realizzato un bioreattore a membrana a fibre cave incrociate adoperante cellule epatiche umane in grado di favorire il mantenimento a lungo termine di epatociti. Il bioreattore è costituito da due fasci di membrane a fibre cave (HFM), uno deputato all'alimentazione e l'altro alla rimozione di cataboliti e prodotti specifici cellulari. I due fasci di fibre sono assemblati in una configurazione incrociata ed alternata in modo da stabilire una distanza l'una dall'altra di 250 μm . Questa configurazione del bioreattore delinea tre compartimenti separati: due compartimenti all'interno del lumen delle fibre cave dove il mezzo di coltura fluisce e un compartimento extraluminale dove le cellule sono coltivate. I compartimenti intraluminali ed extraluminale comunicano tra di loro attraverso i pori della parete di membrana. Il mezzo che fluisce nel lumen delle fibre di alimentazione permea nel compartimento cellulare, dove i cataboliti ed i metaboliti prodotti dalle cellule vengono rimossi dalle fibre cave deputate all'allontanamento dei molecole di sintesi e di scarto cellulari. In questo dispositivo le membrane a fibre cave consentono la compartimentalizzazione delle cellule in un microambiente controllato a livello molecolare ed il trasporto selettivo di molecole verso e dal compartimento cellulare proteggendo le cellule da eventuali sforzi di taglio. Inoltre, le membrane, grazie alla loro geometria intrinseca, offrono un'ampia superficie per l'adesione e la crescita delle cellule in un volume ridotto.

Epatociti umani rappresentano una fonte cellulare ottimale da utilizzare nelle terapie che sono basate sull'uso di cellule, in quanto riflettono più da vicino le condizioni in vivo. In vivo gli epatociti sono altamente proliferativi all'interno del loro microambiente. Tuttavia, quando sono isolati dal loro microambiente e coltivati in vitro, perdono rapidamente le loro funzioni specifiche. Pertanto, è di importanza fondamentale la realizzazione di modelli in vitro in grado di mantenere gli epatociti vitali e funzionali per lungo tempo. Un aspetto critico è la scarsa disponibilità di epatociti umani per cui occorre prendere in considerazione fonti cellulari alternative. Gli studi effettuati in questi ultimi anni indicano come una delle migliori fonti cellulari alternativa agli epatociti le cellule staminali, poiché queste cellule sono ampiamente disponibili possiedono in vitro

un'elevata capacità proliferativa e possono essere differenziate in epatociti. A differenza delle cellule provenienti da animali e delle linee cellulari, le cellule staminali non costituiscono un rischio di trasmissione virale zoonotica o tumorigenicità.

In questo lavoro, il bioreattore a membrana è stato ottimizzato al fine di creare condizioni di coltura per aggregati cellulari come sferoidi e per sistemi organotipici tridimensionali (co-coltura di epatociti e cellule non parenchimali) che garantiscano il mantenimento a lungo termine della funzionalità dei costrutti epatici umani. A tal proposito, le funzioni specifiche epatiche come l'urea, la sintesi dell'albumina e la biotrasformazione di farmaci sono state valutate nel bioreattore. I cambiamenti morfologici cellulari sono stati analizzati utilizzando il microscopio elettronico a scansione ed il microscopio confocale a scansione laser. Inoltre, il consumo di ossigeno delle cellule poste in coltura nel bioreattore è stato continuamente monitorato nel tempo al fine di assicurare un adeguato approvvigionamento di ossigeno.

Gli sferoidi epatici umani, posti in coltura nello spazio extracapillare del bioreattore sono andati incontro ad un processo di fusione che ha portato alla formazione di strutture di maggiore dimensione simili a microtessuti. La fusione degli sferoidi è stata osservata sia tra le fibre che intorno alle fibre simulando il processo che avviene in vivo. Questo modello di coltura, grazie alle sue caratteristiche tridimensionali e all'aumentata interazione cellulare, così come avviene in vivo, ha favorito il mantenimento a lungo termine della vitalità e delle diverse funzioni specifiche epatiche come la sintesi di albumina ed urea ed il metabolismo xenobiotico. Allo stesso modo, nel sistema organotipico, le cellule si riorganizzano formando strutture tissutali simili a quelle del tessuto epatico in vivo. Questo è stato reso possibile grazie al piastramento sequenziale sulle membrane di cellule non parenchimali e parenchimali che hanno formato strutture stratificate tridimensionali simili a quelli in vivo. Il bioreattore che è stato ottimizzato in questo lavoro di tesi fornisce un microambiente di coltura ben controllato da un punto di vista molecolare attraverso l'alimentazione continua di sostanze nutritive, di cui una delle più importanti è l'ossigeno, e la rimozione di cataboliti. Ciò è stato confermato dai risultati relativi alla misura della concentrazione di ossigeno nel mezzo di coltura sia nella corrente in ingresso che in uscita dal bioreattore. In entrambi i modelli di coltura, l'approvvigionamento di ossigeno nel bioreattore è risultato essere sufficiente e significativamente maggiore a quello osservato in condizioni di coltura statica. Inoltre, una nuova fonte di cellule staminali, ovvero le cellule staminali mesenchimali derivate dal fegato, è stata utilizzata: le cellule sono state differenziate con successo in epatociti dopo 24 giorni di coltura, sia nel sistema

statico che nel bioreattore. Tuttavia, il bioreattore ha mostrato una migliore capacità di mantenere la vitalità delle cellule e di differenziare le cellule staminali mesenchimali nel fenotipo epatico, come dimostrato dall'aumento dell'espressione genica di marcatori epatici specifici (ad es. albumina ed il fattore nucleare epatico alfa-4) e dalle velocità di sintesi di urea e albumina.

Il prototipo di bioreattore realizzato su scala di laboratorio ha mantenuto con successo e funzionalmente attivi gli epatociti umani coltivati come sferoidi e in co-coltura con cellule non parenchimali per quasi un mese. Un aspetto importante è stato il differenziamento epatico delle cellule staminali mesenchimali, che rappresentano una potenziale fonte di cellule alternativa agli epatociti umani primari. Tutti questi risultati sono stati ottenuti utilizzando solo cellule umane, che convalidano le prestazioni del dispositivo che è stato sviluppato come sistema epatico bioartificiale da utilizzare in vitro. Questo bioreattore su scala di laboratorio ha un elevato potenziale applicativo che va dagli studi in vitro delle malattie epatiche agli studi di tossicità a lungo termine. Inoltre, può essere realizzato su scala clinica ed applicato come fegato biartificiale per sostituire le funzioni epatiche di pazienti affetti da insufficienza epatica in attesa di trapianto.

Abstract

There is a growing need for a bioartificial liver device for applications ranging from lab-scale platform for drug toxicity testing by pharmaceutical companies to a clinical-scale temporary support device for liver failure patients pending transplantation.

To this end, a crossed hollow-fiber membrane bioreactor was used in conjugation with various human cells to establish long-term cultures. The bioreactor consists of two bundles of hollow fiber membranes (HFM) that are cross-assembled in alternating manner at distance each other of 250 μm . The bioreactor is comprised of three separate compartments: two intraluminal compartments within the feeding and removing HFM, and an extraluminal compartment or shell outside of the fibers that represents the cell compartment. The intraluminal and extraluminal compartments communicate through the pores of the fiber wall. The medium permeates out of the feeding hollow fiber bundle into the cellular compartment. The catabolites and metabolites produced by cells are removed by the other bundle of hollow fibers. The HFM allow the compartmentalization of cells in a microenvironment controlled at molecular level and the selective mass transfer of molecules to and from the cell compartment without causing shear stress. Owing to its intrinsic geometry they provide a wide surface area for the adhesion and growth of cells in a small volume.

Primary human hepatocytes were used as they are the optimal cell source for cell-based therapies, because they most closely reflect the *in vivo* situation. Within their *in vivo* microenvironment, hepatocytes are highly proliferative. However, when they are isolated from their *in vivo* microenvironment and cultured *in vitro* they rapidly de-differentiate losing their liver-specific functions. Therefore, there is an urgent need for the development of *in vitro* models capable of maintaining hepatocytes viable and functional for long periods of time. Moreover, due to the scarcity of human hepatocytes, an alternative cell source needs to be considered. Hepatogenic differentiation of stem cells is considered the best option, since these cells are readily available and can proliferate *in vitro*. Unlike animal cells and cell lines, stem cells do not pose a risk of zoonotic viral transmission or tumorigenicity.

In this work, culture conditions for 3D microtissue spheroids and organotypic cultures (co-culture of hepatocytes and non-parenchymal cells) were optimized in the bioreactor in order to achieve the long-term maintenance of functional human primary liver constructs. Liver-specific functions such as urea, albumin synthesis and drug metabolism were evaluated. Also, morphological

assessment was carried out utilizing scanning electron microscope, confocal laser scanning microscope and light microscope. In addition, oxygen consumption rates were monitored throughout the cultures to ensure an adequate oxygen supply.

The human hepatocyte spheroids cultured in the extracapillary space of the bioreactor fused forming larger microtissue-like structures between and around the fibers that more closely resembles the *in vivo* situation in comparison to single spheroids. By more closely resembling the *in vivo* environment, due to its three-dimensionality and cell–cell interaction, this model supported long-term viability and the maintenance of several liver-specific functions including albumin and urea synthesis and xenobiotic metabolism. Similarly, in the organotypic cultures, cells rearranged forming tissue-like structures that better resemble the *in vivo* liver tissue due to the sequential seeding of cells during the experiment setup resulting in layered structures similar to those seen *in vivo*. The bioreactor provides a well-controlled microenvironment through continuously feeding nutrients, most important of which is oxygen, and removing catabolites. This was confirmed via dissolved-oxygen monitoring in both inlet and outlet of the bioreactor. In both culture conditions, oxygen in the bioreactor was found to be sufficient and significantly higher than those observed in batch condition.

In addition, a novel stem cell source, namely liver-derived mesenchymal stem cells, was successfully differentiated into hepatocyte-like cells after 24 days in culture both in batch systems and HFMBR. However, the bioreactor showed an improved survival as well as differentiation of the mesenchymal stem cells as demonstrated by the increase of gene expression of specific hepatocyte marker (e.g. Albumin and Hepatocyte Nuclear Factor 4 Alpha), and higher urea and albumin synthesis rates.

The lab-scale prototype hollow-fiber membrane bioreactor (HFMBR) realized in this work successfully maintained viable and functionally active human hepatocytes cultured as spheroids and in co-culture with non-parenchymal cells for almost one month. Moreover, it resulted in enhanced hepatic differentiation of mesenchymal stem cells, providing a potential alternative cell source to the scarce primary human hepatocytes. All these results were obtained using human cells only, which validate the performance of the device that was developed as an *in vitro* bioartificial liver platform. This lab-scale bioreactor has various potential applications ranging from basic studies of liver diseases to long-term toxicity studies. Moreover, it can be scaled-up to a clinical scale to be used as BAL device for liver failure patients.

Preface and Scope of Work

Liver disease and the subsequent loss of liver function is an enormous clinical challenge, and is currently the 12th most frequent cause of death in the United States and the 4th most frequent for middle-aged adults (1). Emergence of new liver diseases such as steatohepatitis, absence of a hepatitis C vaccine, and increasing number of hepatocellular carcinoma patients, further worsens the situation (2, 3). Liver transplantation is the only established successful treatment for end-stage liver disease, and currently there are over 119,613 people on the waiting list for a donor organ and of those, there are around 14,525 candidates awaiting a liver transplant (based on United Network for Organ Sharing Organ Procurement and Transplantation Network, UNOS OPTN, December 5, 2016). In 2015, there were 5950 liver transplants performed in the United States. The European Liver Transplant Registry (ELTR) reports that in Europe 137,404 liver transplantations have been performed from 1968 to 2015 (European Liver Transplant Registry).

Various surgical options have been pursued, including living-donor partial transplantation and split liver transplants, in order to expand the supply of livers available for transplantation (4). Despite these surgical advances, organ shortage remains a major hurdle, thus it is unlikely that liver transplantation procedures alone will ever meet the increasing demand. Despite the progress made in supportive care, only 20% of liver failure patients survive without liver transplantation, which increases the survival rates to over 80% (5, 6). Moreover, improving the patient's condition before liver transplantation substantially increases post-transplant survival (1). Lastly, temporary liver support could potentially create time for the diseased liver to regenerate, thereby rendering liver transplantation superfluous. Taken together, there is an urgent need for liver support therapy. For these reasons, many researchers have developed various liver support devices, which can be divided into non-biological (artificial) and biological (bioartificial) systems. Non-biological systems typically use adsorbents and/or dialyzing membranes to detoxify the patients' plasma. Probably the potential of these systems is limited by their lack of metabolizing and synthesizing capacity, as well as the fact that detoxification in these systems is non-specific, which might lead to the removal of beneficial proteins, such as growth factors and clotting factors.

Generally, a BAL system consists of functional liver cells supported by an artificial cell culture material. In particular, it incorporates hepatocytes into a bioreactor in which the cells are immobilized, cultured, and induced to perform

the hepatic functions by processing the blood or plasma of liver-failure patients. BAL devices act as a bridge for the patients until a donor organ is available for transplantation or until liver regeneration (7).

Another important application of the BAL, is the study of drug-induced liver toxicity. Hepatotoxicity is a major cause for drug withdrawals from the market, resulting in huge financial losses for pharmaceutical companies (8, 9). Human-based in vitro models comprising of microsomes, cell lines, primary hepatocytes, and liver slices (10-13) provide additional information to the existing animal models. However, they can be limited by poor stability, and, with the exception of precision cut liver slices, lack the hierarchy and structural components of liver. Monolayer cultures of primary hepatocytes are the most commonly used format for toxicity assessment and provide a suitable model for initial assessment, but are severely hindered by the lack of 3D organization, non-parenchymal cells, and thus cell–cell interactions via contact or paracrine effects.

The aim of this thesis is to refine and further develop more sensitive human in vitro models and methods for longer maintenance of functional hepatocytes by using a crossed hollow-fiber membrane bioreactor that was developed in our laboratory. The work plan constitutes of two different parts:

1) To develop and evaluate new in vitro human liver cell culture models to improve and maintain the hepatic functionality, better extrapolating to human liver biology.

- Development of a 3D liver micro-tissues from hepatocyte spheroids (**CHAPTER 2**).
- A liver co-culture model, incorporating hepatocytes and non-parenchymal cells (sinusoidal and stellate cells) (**CHAPTER 3**).

2) To generate new sources of functional human hepatic cells.

- Establishment of a stepwise, directed, differentiation protocol for hepatic differentiation of human stem cells (Liver Mesenchymal stem cells) (**CHAPTER 4**).

Evaluation of the beneficial effect of the dynamic crossed hollow-fiber membrane bioreactor system (developed in our laboratory) on the prolonged maintenance and enhanced functions of the above-mentioned models was carried out in this context.

CHAPTER 1

State of the art and research scope

1.1 Liver anatomy and physiology

The liver is the largest internal organ in the body and performs several essential functions. These include bile production, plasma protein synthesis, glucose homeostasis and glycogen storage, processing and storage of fats, such as cholesterol, and production of hormones (14). The liver is a highly-specialized tissue that comprises many different cell types, further described below. The liver parenchyma is organized as thousands of small (~0.7 × 2 mm) hepatic lobules, in which hepatocytes form hundreds of irregular plates arranged radially around a small central vein. Peripherally each lobule has three to six portal areas, each of which contains three interlobular structures that comprise the portal triad, namely a branch of the hepatic artery, a branch of the portal vein and a bile ducts (Fig 1.1). The liver is supplied with oxygenated blood from the hepatic artery and venous blood from the portal vein entering the periportal area of the lobule and via branches of small interlobular vessels. Between all of the anastomosing plates of hepatocytes of a hepatic lobule are important vascular sinusoids that emerge from the peripheral branches of the portal vein and hepatic artery and converge on the lobule's central vein. The mixed blood flows through the sinusoids and leaves the lobule via the hepatic central vein located in the center of the lobule. Bile is secreted by the hepatocytes into bile canaliculi, flows in the opposite direction of the blood and empties into the bile ducts that are lined by epithelial cells called cholangiocytes (15). The bile is ultimately secreted into the duodenum where it facilitates the digestion of lipids (14).

The lobule is divided in zones based on the metabolic functions that liver cells have to perform. The concentration of oxygen, nutrients, insulin and glucagon is highest in the periportal area and decreases towards the central vein. As a result of the concentration gradient, hepatocytes in the different zones have different morphology and functions (16). For example, hepatocytes around the central vein have higher density of endoplasmic reticulum and possess the highest levels of enzymes involved in detoxification and biotransformation (17, 18). Substances from orally consumed food and drugs reach the liver via the venous blood from the intestine, which is filtered through the liver before entering the systemic blood circulation. This makes the liver a central organ in metabolism of both endogenous substances, such as bilirubin and ammonia, as well as exogenous substances, like bacterial toxins and alcohol (14). Most pharmaceutical drugs available on the

market today are administered orally which makes the liver a highly-exposed organ for drug toxicity. Due to its central position in the body, the liver also functions as an important immune organ harboring many cells involved in both the innate and the adaptive immune response (19).

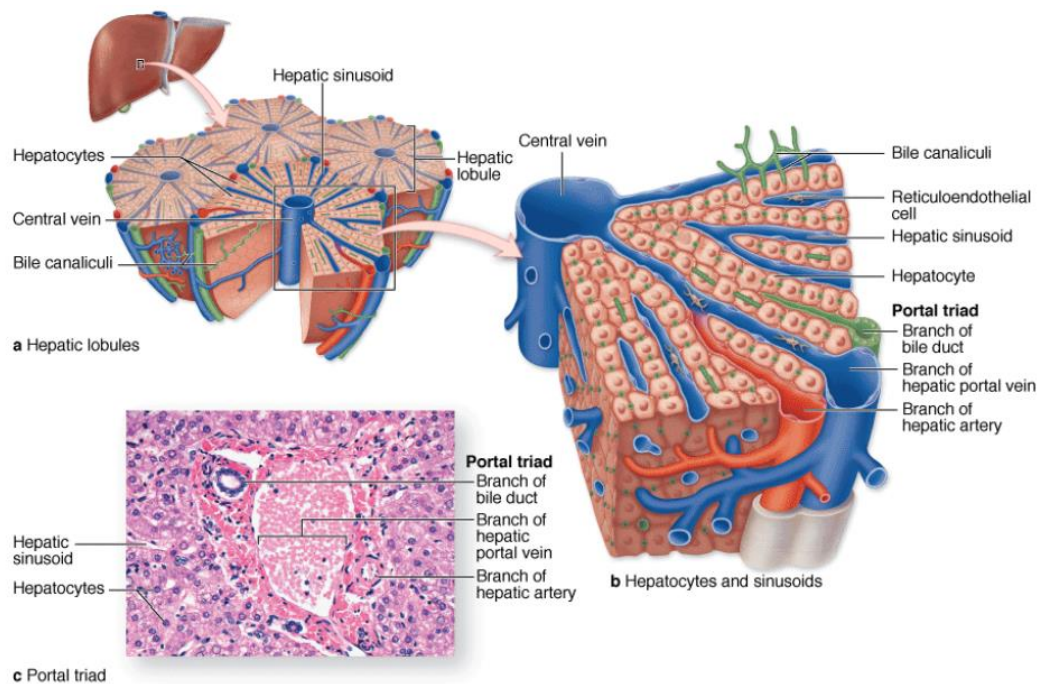


Fig. 1.1. The liver, a large organ in the upper right quadrant of the abdomen, immediately below the diaphragm, is composed of thousands of polygonal structures called hepatic lobules, which are the basic functional units of the organ. **(a)** Diagram showing a small central vein in the center of a hepatic lobule and several sets of blood vessels at its periphery. The peripheral vessels are grouped in connective tissue of the portal tracts and include a branch of the portal vein, a branch of the hepatic artery, and a branch of the bile duct (the portal triad). **(b)** Both blood vessels in this triad branch as sinusoids, which run between plates of hepatocytes and drain into the central vein. **(c)** Micrograph of a lobule shows the central vein (**C**), plates of hepatocytes (**H**), and in an adjacent portal area a small lymphatic (**L**) and components of the portal triad: a portal venule (**PV**), hepatic arteriole (**HA**), and bile ductule (**B**). (X220; H&E). Image taken from (15).

Zonated hepatic functions include glucose metabolism, ammonia detoxification, and the metabolism of drugs and xenobiotics (Fig. 1.2). Glucose metabolism was the first such function to be reported, already in 1977, where gluconeogenesis was found to be mainly periportal; whereas glycolysis was mainly perivenous (20). One of the major roles of the liver is the removal of harmful ammonia arriving from the intestine via the portal vein. Ammonia is first metabolized by periportal hepatocytes, to generate urea. Residual ammonia is then converted by perivenous hepatocytes into glutamine (21). Other zonated processes include: lipid metabolism, with

lipogenesis occurring perivenously and fatty-acid degradation periportally (22, 23). Perivenous hepatocytes are predominantly responsible for drug metabolic activities exhibited by the liver through monooxygenation followed by glucuronidation of the drugs converting them into excretable products (22).

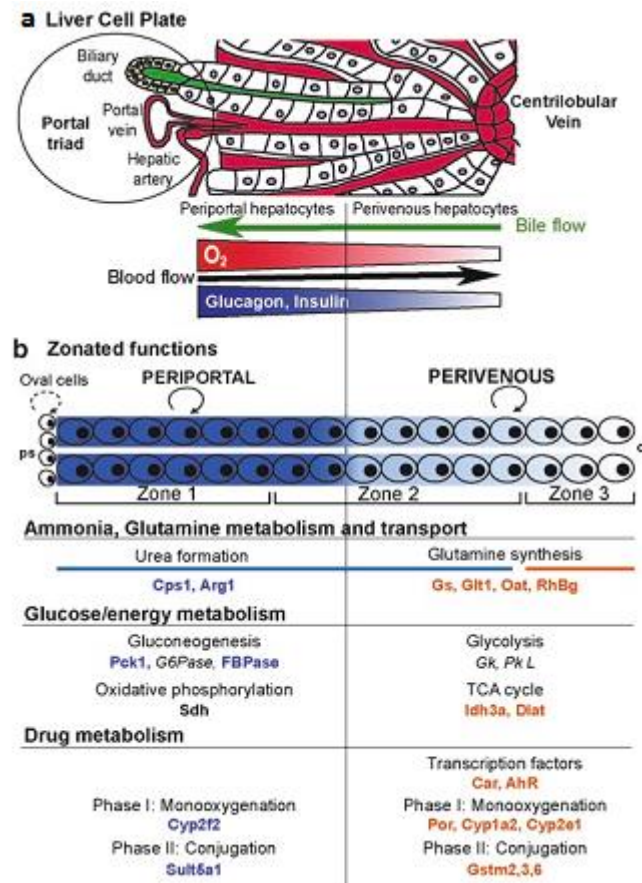


Fig. 1.2 Structure and functions of the zoned liver lobule (a) The liver cell plate, with blood circulation indicated in red. Bile is shown in green and circulates in the opposite direction to blood. The concentration of oxygen and hormones decreases along a continuous gradient from the PP area to the PV area. (b) Zonal functions. The proliferation of hepatocytes is achieved mostly by division of the mature hepatocytes themselves (circled arrow), which do not migrate along the portocentral axis, with proliferation from oval cells observed only rarely (dotted circled arrow). The zoned metabolic systems include the ammonia detoxification system, glucose and energy metabolism, and xenobiotic metabolism. The proteins involved in each type of zonal metabolism are indicated.(24).

Not all hepatic processes are zonal. Some functions, such as serum-protein synthesis, appears to occur in all hepatocytes. Albumin is also synthesized in all hepatocytes, with a higher concentration in the periportal area.

Liver disease usually leads to a variety of life-threatening metabolic and physiologic abnormalities. For example, the absence of these functions leads to bleeding

abnormalities, accumulation of neurotoxins causing hepatic encephalopathy, accumulation of serum ammonia, and jaundice from elevation of serum bilirubin. It is possible to medically support liver disease patients through therapies targeted at features such as portal hypertension and coagulopathy. However, there are no therapeutic strategies effectively replacing the range of affected functions. Thus, an organ transplant has been the only permanently successful therapy to date. This is different compared to other organs, such as the heart and kidneys, in which patients with failing tissues can be supported by pharmaceuticals and machines, without the need for immediate transplantation.

Consequently, efforts have been focused toward the development of liver support systems that could provide temporary support for patients with liver failure. These measures include extracorporeal support devices analogous to kidney dialysis systems, processing the blood or plasma of liver failure patients (25, 26). They range from non-biological-based systems to cell-based therapies, such as BALs. In vivo, the liver exhibits a unique capacity for regeneration, with the potential for full restoration of liver mass and function even after massive damage (27).

However, a major hurdle to the advancement of cell-based therapeutic strategies is the loss of the proliferative capacity and of the liver-specific functions exhibited by hepatocytes once isolated from the in vivo microenvironment (28). Another obstacle is the limited availability of human hepatocytes. Only a limited supply of primary human hepatocytes is currently available from organs deemed inappropriate for transplantation. Thus, significant research efforts are focused on the potential of alternative cell sources, most notably those based on stem cell differentiation and reprogramming.

1.2 Cellular components of the human liver

The hepatocellular parenchyma accounts for 60% of the total cell population and 80% of the total volume of the organ, with the lobular parenchyma representing approximately 93%, the hepatic veins 4%, and the portal triads 3% of the hepatic parenchyma. Non-parenchymal cells comprise 30–35% of the total number of liver cells, but only 6% of the total liver volume. Almost half (40%) of the non-parenchymal cells are fenestrated endothelial cells. The remainder consists of phagocytic Kupffer cells (33%), extraluminal stellate cells (22%), biliary epithelial cells (4%), natural killer cells (1%) (29).

1.2.1 Hepatocytes

The hepatocytes are rich in cellular organelles such as mitochondria, endoplasmic reticulum and Golgi apparatus, a sign of active protein synthesis and secretion from these cells (30). Hepatocytes have a large nucleus and about 25% of the cells are binucleated which often results in polyploidy, suggested to be an important mechanism to restrict liver growth and prolong cell survival (31). The hepatocellular membranes have a complex structure with different membrane sections with different biochemical composition and functional properties: the basolateral section (facing the sinusoids), the lateral (inter-cellular) section, and the apical section facing the bile canaliculi (14). The hepatocytes perform a variety of different functions. They produce bile that is vital for the digestion of lipids. Many serum proteins i.e. albumin and blood clotting factors are synthesized by the hepatocytes and they also regulate the glucose homeostasis in the blood in response to glucagon and insulin signalling. The hepatocytes are also essential for the biotransformation of many endogenous substances, like different serum proteins, lipids and steroids. They also metabolize many exogenous substances, such as alcohol, chemicals and pharmaceuticals. Hepatocytes also play an important role in the hepatic immune response via the production of complement factors and acute phase proteins as a response to cytokine stimuli, like IL-6 (interleukin-6), IL-1 β and TNF α , produced by Kupffer cells and endothelial cells (14). Hepatocytes have also been reported to acquire antigen presenting skills (32) and are generally considered to be both the target and inducer of the innate immune response (33). The liver has a remarkable regenerating capacity both via proliferation of hepatocytes (34) and via activation and differentiation of oval cells (27, 35). Growth factors and cytokines, such as HGF (hepatocyte growth factor), TGF- β , FGF1, IL-6 and TNF α , released by Stellate cells and Kupffer cells, has shown to have hepatoprotective effects and to stimulate liver regeneration (35-37). Additionally, TGF β signaling, which under normal conditions keeps the hepatocytes in a quiescent state, is suppressed during injury (27).

1.2.2 Sinusoidal endothelial cells

The hepatic sinusoids are lined by fenestrated endothelial cells. The basolateral surface of the hepatocyte is separated from endothelial cells by the space of Disse. The fenestration (0.1-0.3 μm) allows efficient transfer of proteins and other macromolecules between the blood and the hepatocytes. The fenestration also facilitates the communication between cells in the sinusoidal lumen and the

hepatocytes as well as other cells in the space of Disse (33). The sinusoidal endothelial cells play an important role in the hepatic immune response as they participate in the clearance of antigens from the circulation by receptor mediated endocytosis, cytokine secretion and by antigen presenting capacities (38). They also collect and present antigens originating from hepatocytes (33). The regulation of endothelial antigen presentation and their role in induction of apoptosis of activated T cells play an important role for the immunologic tolerance in the liver (39).

1.2.3 Stellate cells

The hepatic stellate cells, or fat storing cells, are spindle-shaped cells located in the space of Disse, with extensions into the inter-hepatocellular space. They have an important role in storage and transportation of retinoids (vitamin A compounds) (40) and they have the ability to secrete different components of the extracellular matrix (ECM), like collagen, proteoglycans and laminin, all essential for many hepatocellular functions (41). Stellate cells also play a role in hepatic immunoregulation as they are known to express Toll-like receptors for LPS stimuli (42). Activated stellate cells can amplify an inflammatory response in the liver by secretion of cytokines and chemokines (36) as well as by antigen presentation (43, 44). When activated, the stellate cells become depleted of vitamin A and via fibrogenic activities they start synthesizing large amount of ECM components, including collagen and adhesive glycoproteins (36, 45). Chronic liver injury may lead to overproduction of ECM by the stellate cells which ultimately results in liver cirrhosis (27).

1.2.4 Kupffer cells

Kupffer cells together with lymphocytes constitute the major part of the hepatic immune cells. Kupffer cells are resident liver macrophages with migratory, phagocytic, inflammatory and antigen presenting capabilities, believed to be derived from circulating monocytes (33). The major part of the Kupffer cells are found around the periportal veins where the cells are larger and more active in phagocytosis compared to those found around the central veins (46). Kupffer cells reside in the sinusoids where they are in close contact with passing lymphocytes as well as with the hepatocytes via the space of Disse (33). They constitute the first line of defense and their location provides effective clearance of endotoxins like LPS and other infectious agents (47). Thus, Kupffer cells have important regulatory function in the pathophysiological state of the liver.

1.2.5 Cholangiocytes

Cholangiocytes, or biliary epithelial cells, line a complex tree-like network of conduits that form the biliary tract. Bile acids and organic solutes secreted by hepatocytes into the canalicular spaces between hepatocytes, form the so-called primary bile. This is then transported via cholangiocyte-lined channels to the gall bladder where it is stored. Cholangiocytes modify pH, fluidity and composition of the bile through various secretory and absorptive processes (48). Moreover, they actively participate in reactive and reparative responses to various pathological stimuli during disease states (49). Many drugs that induce a hepatic toxic response or chronic inflammation, result in dysfunction of the bile formation and bile flow, ultimately leading to cholestasis (50).

1.3 Liver support systems

Over the last three decades, liver support systems have been developed to replace orthotopic liver transplantation, or to complement patient care by promoting liver tissue regeneration, or to provide a bridge to liver transplantation. A successful liver support system should provide sufficient detoxification, synthesis, excretion and biotransformation functionality as performed by the liver. They are commonly divided into two main categories: biological and non-biological systems.

1.3.1 Artificial liver support systems

Accumulation of endogenous hepatotoxic substances is conjectured to induce loss of liver function, which in turn gives rise to accumulation of toxins, production of cytokines, and further damage of the liver (25). Artificial liver (AL) devices are typically designed to emulate detoxification functions of the liver through filtration and adsorption mechanisms only, without employing living components.

Two of the most prominent and most used AL devices are Molecular Adsorbent Recirculating System (MARS®; Gambro, Stockholm, Sweden) and Prometheus® (Fresenius Medical Care, Bad Homburg, Germany). The MARS® uses a high-flux hollow-fiber dialysis module made up of albumin impregnated polysulfone membranes. The cut-off of the membranes is limited to 50 kDa to prevent the passage of the proteins and albumin in the patient's blood through the membrane, while membrane-bound albumin act as the acceptor molecule for albumin-bound toxins within the extracorporeal circuit. The toxins are then passed to the 600ml

recirculating 20% albumin solution flowing at constant rate in counter-current flow. The dialysate is sequentially cleansed by a haemodialysis/haemofiltration module (removing water soluble substances) and adsorber columns containing activated charcoal and anion exchange resin (removing most of the albumin bound substances). The regenerated dialysate is recirculated to take up more toxins from the blood (51) (Fig. 1.3a).

On the other hand, Prometheus® is based on fractionated plasma separation and adsorption, in which the patient's plasma is passed through an albumin-permeable polysulfone membrane, which enables the patient's albumin fraction to pass into a secondary circuit in which the direct removal of albumin-bound toxins by different adsorbers (that is, anion exchanger and neutral resin) takes place (Fig. 1.3b). In addition, conventional, high-flux dialysis is performed within the primary (blood) circuit (52).

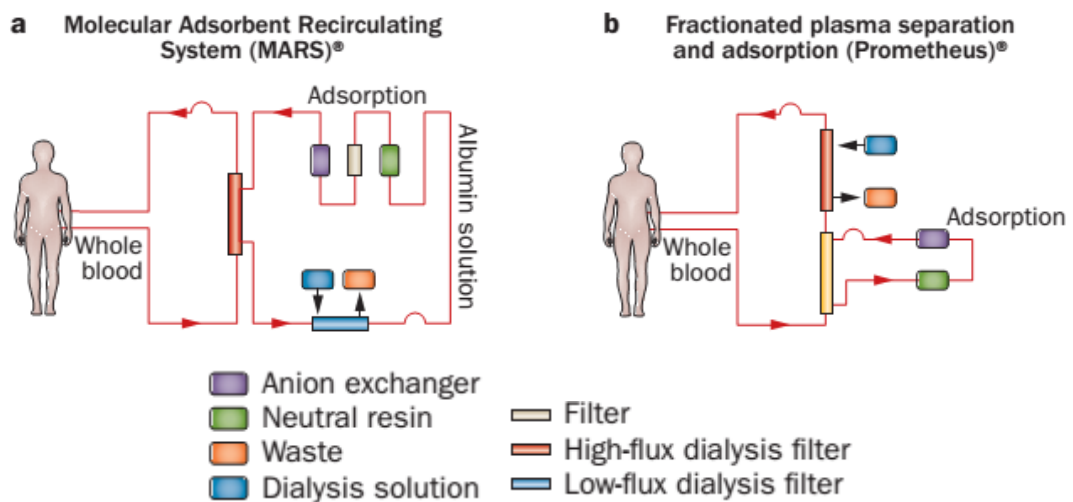


Fig. 1.3. Artificial liver support systems. The hypothesis that the hepatocellular dysfunction present in the clinical syndrome of liver failure is primarily caused by the accumulation of toxins not cleared by the failing liver is addressed by filtration and adsorption devices. a) Molecular Adsorbent Recirculating System (MARS®; Gambro, Stockholm, Sweden). b) Fractionated plasma separation and adsorption (Prometheus®, Fresenius Medical Care, Bad Homburg, Germany). Adapted from (52).

ALs are merely based on physico-chemical mechanisms and thus lack synthetic and biochemical functions of the liver. Additionally, hepatic detoxification inside the body is not limited to elimination of albumin-bound toxins. Moreover, Recent clinical

trials showed no survival benefit of extracorporeal liver support therapy compared with standard therapy (26).

In search for a device that provides a larger complement of important liver functions, including synthetic and metabolic processes, biohybrid support devices incorporating living hepatic cells have been developed (53).

1.3.2 Bioartificial liver support systems

Cell-based therapies have been extensively studied to overcome the limitations of the ALs and expand the spectrum of hepatic functions available. Among these therapies are isolated cell infusion via the hepatic portal vein for the transplantation of hepatocytes derived from primary or stem cells, extracorporeal bioartificial liver (BAL) devices integrating liver cell culture in a bioreactor, and tissue-engineered graft implantation. It is a significant challenge to sustain and extend hepatocyte function *in vitro*. Liver functionality is regulated by soluble mediators, interactions between cells and extracellular matrix (ECM), and interactions between cells, which all represent types of microenvironmental signals. Therefore, to gain a comprehensive understanding of these regulating processes, different *in vitro* hepatic culture models have been created, such as perfused whole-organ and wedge biopsies, precision-cut liver slices, isolated primary hepatocytes, immortalised liver cell lines, and isolated organelles (54).

The BALs have great potential for the creation of a short-term support and framework for drug assessment and *in vitro* research on hepatic cultures. Various different hepatic functions, including detoxification, metabolism and synthesis, could be undertaken by BALs owing to the fact that they integrate functional hepatocytes.

To preserve the phenotypic functions of cells, it is expected that the *in vivo* microenvironment will be replicated by *in vitro* cell cultures. External cues inserted in the microenvironment of isolated hepatocytes with regard to time as well as space determine how viable and functional those hepatocytes are (55). The clinical success of a BAL device depends on its ability to provide suitable mass transport, lengthen cell viability and functionality, and be amenable to scaling-up to a size of clinical relevance.

A number of limitations are presented by the basic static monolayer (2D) cultures based on petri dish: (i) nutrient depletion and aggregation of cellular metabolic products cause ongoing temporal transformations in the cellular microenvironment; (ii) oxygen is poorly soluble and has long diffusion length, which is why it occurs in

restricted supply; (iii) unstable interactions between cells and between cells and the ECM; (iv) lack of comparison between the smooth, 2D substrate and the *in vivo* settings; and (v) the lack of permeability of the substrate causing unidirectional mass exchange.

The various BAL designs that have been proposed to address these limitations can be categorised into four major groups, namely, flat plate systems, hollow fibre membrane bioreactors (HFMBR), perfused packed bed/scaffold systems and suspension/encapsulation-based reactors (Figure 1.4). Furthermore, research has also been conducted recently on microfluidic systems and microfabricated reactor systems for *in vitro* applications (56).

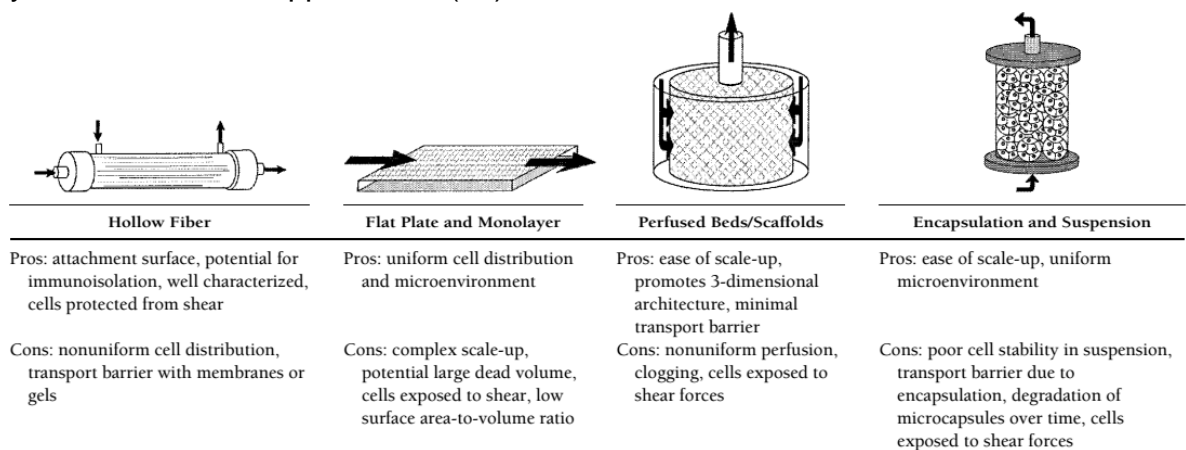


Fig. 1.4. Schematics of cell-based bioreactor designs. The majority of liver cell-based bioreactor designs fall into these four general categories, each with inherent advantages and disadvantages. Adapted from (57).

1.3.2.1 Flat-Plate System

An additional approach towards overcoming the above-mentioned limitations has taken the form of various strategies explored in relation to bioreactors. Although considerable improvement in mass transfer can be achieved with perfusion, a considerable obstacle is posed by the shear stress imposed on the cells, which has to be kept within a range that is acceptable to the hepatocytes. Membrane bioreactors include flat-plate as well as hollow-fibre systems (Figure 1.5).

Dynamic culture systems gain numerous advantageous features as a result of membrane integration in flat-plate bioreactors. Membranes enable system arrangement into clear-cut sections and mediate the passage of nutrients and oxygen to the cellular part whilst at the same time preventing fluid shear. Furthermore, membranes permit selective mass transfer of molecules to and from

cells in every direction by supporting one or both cellular layer surfaces. Moreover, separate application of oxygen gradient from the fluid flow is made possible by membranes with oxygen permeability. Unlike smooth solid plastic, a 3D substrate closely resembling the *in vivo* one is provided by porous membranes, which is helpful for research on cellular interaction (58).

Among the additional improvements that have been considered are monolayer-based systems with applicability to flat-membrane bioreactors as well, including co-cultures (59) and sandwich cultures (60) for enhancing cell-cell and cell-ECM interactions, and substrate micropatterning (61) for cell morphogenesis studies. A radial-flow bioreactor consists of a stacked microfabricated grooved substratum, with hepatocytes being protected against flow shear stress by the grooved glass plate (62). Meanwhile, among the various existing *in vitro* hepatocyte culture models, sandwich culture is believed to be one of the ideal ones. However, the application of sandwich culture in BAL bioreactor design is hampered due to several technical difficulties, such as cell housing capacity that is not as high as other configurations and arduous bioreactor construction process, that need to be addressed in future bioreactor design.

1.3.2.2 Hollow-Fiber System

The main type of bioreactor configuration is the hollow-fibre system. Tissue engineering applications for cell-based treatments (63) including BAL devices (64) and large scale cell cultures (65) widely employ hollow-fibre membrane bioreactors (HFMBR).

An HFMBR is made up of a cluster of hollow fibres (HF) with partial permeability put in an external housing, like ultrafiltration or dialysis applications. Each configuration can fulfil a distinct function, according to the application and type of cell employed. In general, the cells are bound to the HF external wall, with flow of medium within the lumen of the fibres that exchange nutrients and other elements, comparable to a network of blood capillaries. Conversely, it is possible to seed the cells into the HF lumen with module operation under various patterns of flow (66). In addition, multibore system (67) alginate immobilization (68) and three-compartment multi-coaxial system (69) are among the other configurations of greater complexity that have been proposed.

In terms of the restrictions related to diffusion length, mass transfer rates for nutrient supply as well as for waste elimination can likely be enhanced by HFMBRs.

Cell protection against shear and air-liquid interfacial stresses is ensured by compartmentalisation, which keeps the cells apart from the direct fluid flow.

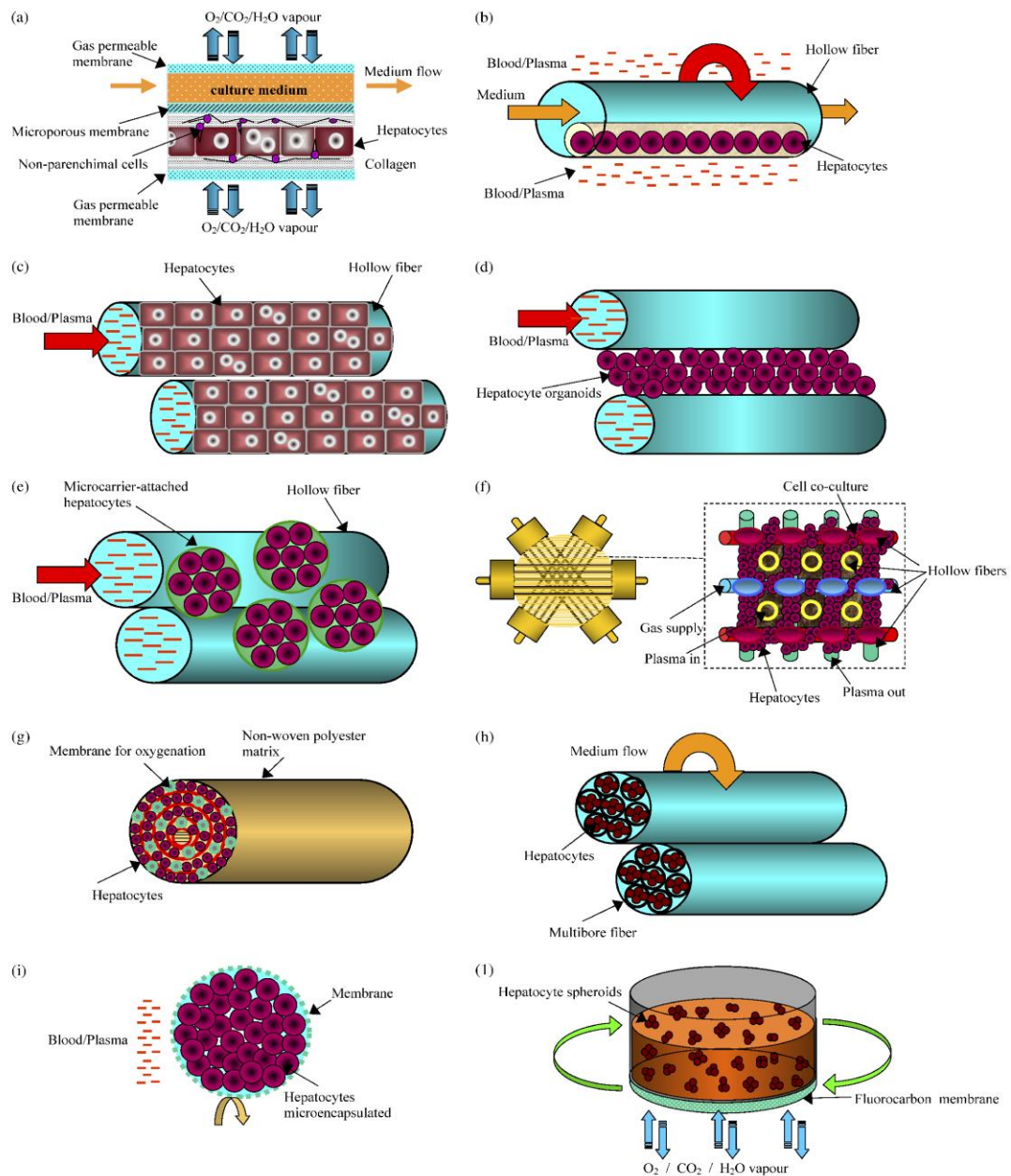


Fig. 1.5 Configuration of membrane bioreactors using hepatocytes cultured (a) between flat-sheet membranes; (b) entrapped in a three-dimensional contracted gel matrix inside of hollow fibre membranes; (c) outside of hollow fibre membranes in monolayer; (d) outside of hollow fibre membranes organised in organoids; (e) outside of hollow fibre membranes attached to microcarriers; (f) in a network formed by four capillary membranes with different functions; (g) in a spirally wound non-woven polyester matrix inside of hollow fibres; (h) in the intraluminal compartment of a multibore fibre bioreactor; (i) in microcapsules and (j) in a rotating-wall gas-permeable membrane system. Image taken from (70).

Furthermore, metabolite transport is enhanced and dead volume is reduced by HF designs because, by comparison to flat-plate designs, they offer a greater ratio of surface area to volume. On the downside, the use of closely packed HFs makes it difficult to seed cells into the bioreactor homogeneously (57).

1.3.2.3 Perfused Bed System

Packed bed refers to a hollow vessel that is filled with packing materials. In application of EBAL, this configuration can be used to fill matrix for hepatocyte attachment and perfused with medium or patient's blood/plasma. Various packing materials for hepatocyte entrapment, such as micro-channeled polyurethane foam (71), polyvinyl resin cubes (72), alginate beads (73), porous hydroxyapatite beads (74), and polyester fabric cell scaffold (75) were explored. Packed bed reactors offer improved mass transfer by allowing direct contact of cells on microcarriers or packing material with the perfusing media (76, 77). A single monolayer culture is easily perfused, but a series of stacked plates may introduce shunting through regions of low resistance. Uniform perfusion of packed bed reactors is a classic engineering problem. Distribution of fluid flow is greatly dependent on the characteristic of the packing material. Larger, rigid particles will yield well distributed flow but a decreased surface area for cells, whereas smaller, porous packing will result in clogging and fluid channeling (78). A packed bed reactor built around a micro-channeled scaffold is an example of one designed explicitly to reduce heterogeneous perfusion and improve the transport characteristics of the devices (79).

1.3.2.4 Encapsulation System

Some designs use encapsulated cells in perfusion systems, which provide immunoisolation, but also increases diffusion resistance (80, 81). Encapsulation-based bioreactors are fairly easy to scale-up and provide a uniform microenvironment for the cells; however, encapsulation presents a diffusion barrier that can lead to cell death in the middle of the beads as well as the degradation of the microparticles over time resulting in exposure of cells to shear stress. Various materials have been used for hepatocyte encapsulation, including hydrogels (80), alginate (81), and copolymer such as hydroxyethyl methacrylate-methyl methacrylate (HEMA-MMA) (82). In some systems, hepatocytes form spheroids before entrapped into a capsule (83, 84), because there are evidences showing that spheroid enhances cell-cell interaction (85), and facilitates the formation of bile-duct structure between cells to improve cell functions.

1.3.2.5 Microfluidic Chip Based System

Microfabrication based microfluidic systems for hepatocyte culture has emerged as a promising area for various hepatocyte in vitro applications including BAL. The fine control of hepatocyte microenvironment, which is essential to maintain hepatocyte differentiated functions, is made possible at microscales in microfluidic system. The small fluid volume in microfluidic perfusion allows more efficient mass transfer in terms of delivery and removal of soluble substance (86). Lee *et al.* (87) created an artificial liver sinusoid with a microfluidic endothelial-like barrier and rat or human hepatocytes can be cultured within the barrier. Hepatocyte viability and drug metabolism functions can be maintained for 7 days in the system. The first attempt to design a microfluidic system based bioreactor for BAL was by Leclerc *et al.* (88). Ten microfabricated polydimethylsiloxane PDMS chips were stacked to constitute four interconnected cell culture chambers and one oxygen supply compartment. The microfluidic bioreactor can achieve high cell seeding density of 30–40 million cells/cm³ with efficient mass transfer, which demonstrated its potential of scalability for BAL application. Recently, Pang *et al.* managed to design a 3D scaffold comprised of 43 culture chambers assembled in a symmetrical pattern on 3 layers. The seeding density was further increased to reach 90 million cells/cm³ (89).

1.3.3 Hepatocyte transplantation

It involves the infusion of donor hepatocytes into the liver of the patient or even into ectopic sites such as the spleen. In principle, the injected cells should be engrafted and proliferate to repopulate the recipient liver restoring its functions (52). This technique was used to temporarily support patients with inborn metabolic liver diseases, such as Crigler–Najjar syndrome type I (90) and urea cycle disorders (91). However, with respect to acute liver failure, currently reported clinical trials have shown inferior results (92). The limited supply of viable hepatocytes, insufficient integration and survival of transplanted hepatocytes, and inadequate immunosuppressive therapy for hepatocyte transplantation patients need to be addressed before establishing this technique as a routine clinical treatment (93).

1.3.4 Repopulation of decellularized liver

One approach to engineer a vascularized, functional tissue is the decellularization and recellularization of livers. During decellularization, cells and other immunogenic molecules are removed from tissue or organs to leave the extracellular matrix. Resident cells generate the extracellular matrix which preserves the complex 3D microanatomy of the liver, including its vascular and biliary system frameworks. Furthermore, the extracellular matrix is ideally suited for repopulation with cells, their subsequent engraftment and migration to their specific niches (94). The recellularized grafts are then perfused at 37°C in bioreactors, using either culture media or heparinized blood. In vivo implantations of engineered hepatic tissue have been thus far limited to animal studies, with the first study reported in 2010 (95) in which rat livers were decellularized then recellularized with rat hepatocytes. The grafts were then implanted in rats with arterial blood flow from the renal artery for up to 8 h. Later, segments of decellularized porcine livers were 'humanized' via infusions of human fetal hepatocytes with co-cultured human fetal stellate cells followed by perfusion in vitro for up to 13 days (96). Inside the extracellular matrix, the human fetal hepatocytes differentiated into hepatocyte-like cells with some mature hepatocyte properties and bipotential progenitor cells. Although in general implantations of recellularized livers in humans seem possible, various issues need to be addressed. One of the major obstacles is the reconstruction of vascular network via re-epithelialization. This is considered the limiting step since contact between the extracellular matrix proteins and blood components leads to the formation of blood clots. Thus, implantations of recellularized grafts will not be possible until the vasculature is completely re-epithelialized or thrombogenesis within the graft is prevented in a different manner without deleterious systemic effects on the host (52). However, the fact that the vascular extracellular matrix frame is conserved during decellularization is possibly the biggest advantage of this technique in comparison to the other approaches. Preclinical experiments and clinical trials are needed to define relevant quality standards to predict the in vivo behaviour of decellularized tissue of different species in humans.

1.3.5 Organ printing

3D bioprinting is a cutting-edge technique that allows for precise engineering of complex parenchymal organ constructs. 3D organ printing can be defined as layer-by-layer additive biofabrication using liquid bioink (cell suspensions) or self-

assembling cellular building blocks (spheroids) (52). 3D spheroid printing represents scaffold-free tissue engineering which enables the precise arrangement of different cell types and other biologic materials (such as extracellular matrix components and growth factors) within organ constructs (97). The spheroid printing technique is based on tissue fusion, a process driven by tension forces between fluids that is observed during embryonic development (98). Tissue fusion means that spheroid blocks of different living cell types that have been placed closely together by a bioprinter melt together to finally represent one entity. Although no reports on printed perfusable hepatic constructs have been published so far, some interesting experimental results demonstrate the potential of 3D printing techniques. The NovoGen MMX Bioprinter™ (Organovo Holdings, Inc., San Diego, CA, USA) was used to print metabolically active 3D hepatic tissue that were stable for over 40 days in vitro (99). Furthermore, compartment-specific organization in a rudimentary microanatomy was shown for hepatocytes, hepatic stellate cells and endothelial cells.

Despite the promising prospect of this technique, these hepatic micro-organs are not designed as organ supportive therapy options. Several hurdles need to be overcome before such a technique can be used to print clinical-scale functional livers. Detailed knowledge of the liver's micro-anatomy, and software to convert this information into reasonable blueprints are crucial. Furthermore, complex bio-mathematical models to predict the behaviour of biological materials during and after printing are needed. Currently available printing hardware can print a tissue of 1 cm³ in 27 h (100). Thus, enhancement of available printing hardware to quickly process living cells is a determining step, as the printing process affects the viability of cells. Moreover, maturation processes further limit the scalability of printed tissue, as it can take months until engineered tubules of printed spheroids are functional and perfusable (101). Thus, sustaining the viability of parenchymal cells during maturation and essentially accelerating maturation are fields of particular interest, especially as most experiments so far have been performed with young animal cells—human primary cells are even more complex to handle in vitro (102).

1.3.6 Induced organogenesis

Induced organogenesis is a completely new approach that focuses on the generation of functional, implantable organs using stem cells (alone or with primary cells). These cells are induced in vitro to initiate differentiation towards a certain fate through manipulating extracellular matrix and growth factors, followed by in

vivo implantation to allow for maturation of the differentiated cells (103). Recently, human induced pluripotent stem cells were differentiated into endodermal cells and co-cultured with human mesenchymal stem cells and hUVECs in vitro (104). After self-organization of these cells into a kind of vascularized liver bud, they were implanted into immune-deficient mice. Interestingly, the in-vitro generated liver bud was shown to integrate into the vascular system of the recipient only 48 h after implantation. Furthermore, the liver bud matured to functional tissue, resembling adult liver tissue and was able to rescue mice in a drug-induced lethal liver failure model. These encouraging results open up a new field for further research and demonstrate that experimental mimicking of organogenesis might lead to liver support therapies in the future.

1.4 Cell source as critical issue for BAL devices

1.4.1 Primary hepatocytes

Primary human hepatocytes are ultimately the preferred cell type for cell-based therapies, and the development of primary hepatocyte-based approaches is the focus of substantial ongoing research. Within their in vivo microenvironment, hepatocytes have high proliferative capabilities. Following partial hepatectomy of two-thirds of the human liver, the residual mature hepatocytes are able to proliferate and restore the lost liver mass (105). However, primary hepatocytes are phenotypically instable and when they are isolated from their in vivo microenvironment and put in 2D cultures they rapidly de-differentiate into a population of adult liver progenitors (106), losing many of their liver specific functions, in particular CYP enzyme levels (107). In addition, 2D culture limits the survival of the cells to only 1-2 weeks (108). Various attempts have been made to prolong the hepatocyte survival in vitro, for example by culturing primary hepatocytes in a sandwich culture of collagen or matrigel, the hepatocyte life span, morphology and specific hepatic functions can be preserved for longer period of time (108, 109). Unfortunately, the low availability of fresh human liver samples compromise the use of primary hepatocytes in routine testing. Moreover, the resected livers most often originate from medicated patients that may severely affect cell viability and specific functions. Regarding donated livers, the patients have often been subjected to various pharmaceuticals, e.g. for the treatment of brain injury, again potentially affecting the expression of various drug metabolizing

enzymes (110). Cryopreserved hepatocytes are often used as they are available and phenotypically characterized which facilitates their use in routine research(111). Moreover, pooled cells from several donors are available which reduce inter-donor variability. However, these cells are expensive and share the same limitations as freshly isolated hepatocytes regarding loss of liver specific functions in culture.

Primary porcine hepatocytes, have been widely used as the cell source for hybrid artificial livers. Porcine hepatocytes exhibit liver-specific functions including biotransformation functions, ammonia detoxification, and synthesis of urea and albumin (112). Although these cells can be readily obtained in large quantities and demonstrate similar functions and therapeutic effects to human hepatocytes, their major drawbacks are the risk of xenogeneic infections such as porcine endogenous retrovirus and the lack of metabolic compatibility (113).

1.4.2 Cell lines

Immortalized hepatocyte cell lines, such as HepG2 (human hepatoblastoma) (114), the HepG2-derived line C3A (115) HepLiu (SV40 immortalized) (116), immortalized fetal human hepatocytes (117) and HepaRG (human hepatoma) (118) have been utilized as readily available alternative to primary hepatocytes. The advantages of using established cell lines include the ability to culture large quantities of cells for an extended period of time and the ability to control the degree of hepatocyte functions that are displayed (119). However, due to lack of the full functional capacity of primary adult hepatocytes, and the risk of transmittance of oncogenic factors to the patient, the use of these cell lines in a clinical setup remains a concern (57). Thus, as a potential precautionary measure, the use of conditionally immortalized lines and the incorporation of inducible suicide genes have been considered (54).

1.4.3 Stem cells

An independent approach to generate hepatocytes for therapeutics is to use stem and/or progenitor cells, which may be sourced from various tissues and have a high proliferative capacity. In principle, such cells could be amplified, differentiated into various cell types, and used in diverse applications (120). Recently, researchers have been attempting to identify small molecules that can potentially induce the maturation of hepatocyte-like cells to enhance the chance for using these cells in a clinically relevant setup (121).

1.4.3.1 Embryonic stem cells

The breakthrough for stem cell research came about 30 years ago with the first successful isolation of an embryonic stem cell line from a mouse embryo (122, 123). In 1998, the first generation of an in vitro, multi-passaged, culture of human embryonic stem cells (hESC) was reported (124) and since then many different protocols have been developed for the establishment, propagation and characterization of hESC. These cells are pluripotent and can generate all three germ layers, thus capable of differentiating into any kind of cell in the human body (124, 125). With these unique properties, these cells provide a highly interesting model system for basic research on embryonic and organ development, as well as a hepatic cell source for drug discovery and toxicology studies (126, 127). In the future, these cells might also be used in clinical therapies (128). During the recent years, much effort has been put into the development of effective protocols for hepatic differentiation of hESCs, largely based on what is known about the embryogenesis (129-131). Various directed differentiation strategies have been applied to hESC and iPSC cultures, and have yielded populations that exhibit some functional and phenotypic characteristics of mature hepatocytes, thus termed "hepatocyte-like cells" (130, 132, 133). Yet, hepatocyte-like cells resemble foetal hepatocytes rather than mature adult cells as evident by their characteristics, such as distinctive cytochrome P450 activities as well as expression of foetal genes such as α -fetoprotein (134). Moreover, all differentiation protocols result in highly variable functionality within the cell population and cells begin to lose hepatic characteristics after a few days much like standard culture conditions. Researchers are developing methods to overcome these disadvantages of functional variability, low expression levels, and loss of functionality over time, however embryonic stem cells are a controversial source of cells for scientific research and this is not likely to change in the near future. A different source of stem cells could eliminate this ethical controversy.

1.4.3.2 Hepatic somatic stem cells

Somatic stem cells have a more limited differentiation potential compared to hESCs but have an important role in tissue homeostasis and injury repair in the multicellular organism (135). The presence of hepatic stem cells (oval cells) were first discovered in fetal mice livers (136) and was later also isolated from human adult livers (137). Oval cells are multipotent stem cells that can give rise to both hepatocytes and biliary cells (35). Hepatic oval cell lines have been generated that retain the progenitor cell features expressing markers for both cholangiocytes and

hepatic progenitors after long term cultivation and serial passages (138). These cells might thus serve as an expandable hepatic cell source for research and for cell-based therapy (139, 140). Recently, progress has been made in identifying tissue-resident mesenchymal stem cell-like populations that reside in human liver (141, 142), further investigation into the role of these cells in normal liver physiology and repair is needed to determine whether these cell populations represent a clinically relevant source of hepatocytes.

1.4.3.3 Induced pluripotent stem cells

In 2006, a Japanese research group successfully reprogrammed adult mouse fibroblasts into induced pluripotent stem cells (iPSC) by introducing four transcription factors: cMyc, Oct3/4, Sox2 and Klf4, by retroviral transduction (143). A similar approach was also successfully performed with human fibroblasts (144) and later using other cell types from both human and mouse (145). The iPSC are stem cell-like regarding morphology and characteristics, such as pluripotency and genetics, expressing a number of stem cell biomarkers (145). Several groups have subsequently been able to successfully generate hepatocyte-like cells from iPSC (133, 146, 147). The iPSC technology is promising with a future potential in patient- and disease-specific therapy (145). However, in order for these cells to be used in a clinical application several important issues have to be addressed, such as somatic origin memory, donor dependent variations, low reprogramming efficiency, risk of potential teratoma formation, safety concerns regarding transduction delivery methods and the presence of transgenes, like oncogenes (148).

In light of this progress, the strategy of using stem cells as a source for generating hepatocytes as a cellular component in BAL devices holds great promise.

1.5 Oxygen supply

Owing to their various metabolic, synthetic and regulatory activities, hepatocytes have an oxygen consumption rate more than ten-fold higher compared to the majority of other cells, triggering an oxygen concentration gradient along the sinusoids (149). It is believed that metabolic function distribution along the sinusoids influences the concentration distribution of oxygen, hormones, nutrients and ECM molecules (54). There are three metabolic areas along lobule hepatic sinusoids that have been distinguished, namely, the periportal zone surrounding the portal vein and hepatic arteriole (zone 1), an intermediate zone 2, and the

perivenous/pericentral/centrilobular zone surrounding the central vein (zone 3). Hepatic metabolism in each zone can be discerned based on the fact that, along the sinusoids, there is a reduction by about half in partial oxygen pressure, which declines from around 60 to 65 mmHg (84-91 $\mu\text{mol/L}$) and from 30 to 35 mmHg (42-49 $\mu\text{mol/L}$) in zones 1 and 3, respectively (150, 151).

It is extremely difficult to replicate *in vitro* the delicate *in vivo* oxygen conditions. To prevent hypoxia, hepatic cultures must have enough oxygen supplies; ensuring these supplies is a problem because oxygen is poorly soluble in culture medium and does not diffuse deep enough in hepatic tissues. By contrast, cells may suffer oxidative effects due to hyperoxic oxygen tensions, which may affect the viability and functionality of hepatocytes. Moreover, it would be useful to induce a concentration gradient compatible with the physiological range in the bioreactor's hepatic mass.

There is a lack of consensus regarding the ideal oxygen partial pressure range for *in vitro* applications. The contradictory results obtained by different studies regarding oxygen tension and uptake rate were reviewed by Wang et. al. (152), concluding that the *in vivo* conditions instead of intracellular values should be replicated by hepatocytes' pericellular oxygen partial pressure.

There are two major approaches to enhance oxygen supply and prevent hypoxic/hyperoxic conditions: (i) membranes with oxygen permeability commonly used in AMC-BAL and MELS devices to improve oxygen supply and achieve local induction of oxygen concentration gradient, and (ii) addition of advection to improve mass transfer.

There are currently no devices suitable for use in regular clinical practice for treating hepatic failure cases, even though significant endeavours have been made to create a BAL capable of replicating the *in vivo* liver function. Absence of an optimal source of cells, restrictions on oxygen supply, and creation of a cellular environment resembling the *in vivo* one are among major obstacles that are yet to be overcome. Designing a functionally active BAL system based on development of highly differentiated hepatocytes from progenitor or stem cells should be a priority for future research. An additional line of inquiry worth pursuing is use of hepatocyte co-culture with other types of hepatic cells by applying strategies conducive to the formation of microvessels and bile canaliculi. Moreover, greater attention should also be paid to the design of membrane devices capable of supporting hepatic functions by supplying the required biological, chemical and mechanical demands or stimuli.

1.7 Bioreactor configurations in bioartificial liver designs

So far, only few BAL systems have reached clinical trials, while various designs are explored in vitro as dynamic hepatic culture systems. The majority of BALs in clinical trial (Table 1) as well as many in vitro systems are based on hollow fiber bioreactors, further demonstrating their significance in these applications.

1.5.1 Bioartificial liver systems in clinical trial

The HepatAssist™ Device is among the popular BAL systems that have made it to the stage of clinical trials. This device involves use of HF membrane-based modified dialysis cartridge for preservation of porcine hepatocyte cells. After separation, the patient's plasma passes sequentially through an activated charcoal adsorber, oxygenator and bioreactor (153). The results of a randomised controlled trial (RCT) on 171 patients with acute liver failure revealed that the HepatAssist device increased survival at 30 days by 71% compared to 62% by conventional medical treatment. Additionally, the HepatAssist group also increased survival of patients with fulminant or sub-fulminant liver failure. However, no other RCTs have so far been conducted on BAL support systems, with only small-scale studies investigating other types of BAL systems (25).

The Extracorporeal Liver Assist Device (ELAD®) (Vital Therapies Inc., San Diego, CA, USA) devised by Sussman et. al. was among the first clinical devices to employ HF membranes. The concept behind the flow circuit of this device is similar to HepatAssist, with the exception that it uses C3A human hepatoblastoma cell lines (115, 154).

A different approach was adopted by Chamuleau et. al. in the creation of the Academisch Medisch Centrum Bioartificial Liver (AMC-BAL), a device made up of non-woven polyester matrix and HFs placed in a housing. Oxygen is supplied and carbon dioxide eliminated by the HFs dispersed in the matrix and the culturing of cells on the matrix is supported by a three-dimensional framework. Although porcine hepatocytes were employed at first, the device was altered to permit the use of immortalised foetal liver cell lines. What makes AMC-BAL different is that there is direct contact between the patient's plasma and the cells following plasmapheresis, which improves cell-plasma mass transfer (155).

Meanwhile, a hemofilter consisting of newly isolated porcine cells forms the basis of the Bioartificial Liver Support System (BLSS) (156), which stands out from other devices in that it involves perfusion of the whole blood, not just plasma, and cells are in suspension and move in the extra-capillary space (ECS) (157).

Among the HF bioreactor models of the greatest complexity but also greatest appeal is the Modular Extracorporeal Liver Support (MELS) created by Gerlach et.

al. It is made up of three distinct but interwoven HF clusters, namely, two clusters of hydrophilic polyethersulfone HFs for plasma perfusion, with semi-interstitial flow of culture medium between them, and a cluster of hydrophobic multilayer HFs for local oxygen supply. Primary porcine or primary human liver cells that can form structures resembling tissues between HFs are present in the bioreactor into which the plasma is perfused (158).

In Radial-flow bioreactor RFB, 250-300 g of 90% viable porcine hepatocytes were seeded on woven-nonwoven polyester fabrics sandwiched between 2 polyester screens (159). Culture medium was perfused radially from the center to the periphery while passing through the cells entrapped within the microfibers.

Table 1.1. Bioartificial Liver systems currently in clinical trials

BAL system	BR type	Culture Technique	Cell type	Cell source	Cell amount	Perfusion medium	BR flow rate (ml/min)	oxygenation
HepatAssist	HF	Microcarrier- attached aggregates	Porcine	Cryopreserved	$5-7 \times 10^9$	Plasma	400	Before bioreactor
ELAD	HF	Hollow fiber attached aggregates	Human, cell line	C3A	200-400 g	Blood / plasma ultrafiltrat	15-200	Before bioreactor
AMC	HF	Polymeric matrix attached aggregates	Porcine	Freshly isolated	10^{10}	Plasma	150	Inside bioreactor
BLSS	HF	Collagen gel entrapped	Porcine	Freshly isolated	70-120 g	Blood	100-250	Before bioreactor
MELS	HF	Tissue-like organoid	Porcine /human	Freshly isolated	≤ 600 g	Plasma	100-200	Inside bioreactor
TECA-HALSS	HF	Cells in outer space of hollow fiber	Porcine	Freshly isolated	$1-2 \times 10^{10}$	Plasma	Not Reported	Before bioreactor
HEBAL	HF	Cells in outer space of hollow fiber	Porcine	Freshly isolated	1×10^{10}	Plasma	Not Reported	Not Reported
RFB	PB	Microfiber entrapped aggregates	Porcine	Freshly isolated	250-300 g	Plasma	200-300	Before bioreactor

*HF= Hollow Fiber

*PB= Packed Bed

1.5.2 Bioreactor designs for *in vitro* hepatic culture studies

Research has assessed the potential of different bioreactor designs for *in vitro* dynamic hepatic cultures for use as BAL devices or drug testing platforms in the pharmaceutical field. To shed more light on bioreactor design and different related design features, several such designs of varying configuration are discussed in the following part.

An oxygen-permeable, flat-membrane bioreactor was developed by De Bartolo et al. (160) (Fig. 1.6a). The culturing of human hepatocytes was undertaken between two semi-permeable fluorocarbon flat membranes. The cells attached to the bottom membrane and received oxygen through both surfaces. To assess hepatic functionality, urea and albumin were synthesised and diclofenac biotransformation was undertaken, with preservation for 32 days.

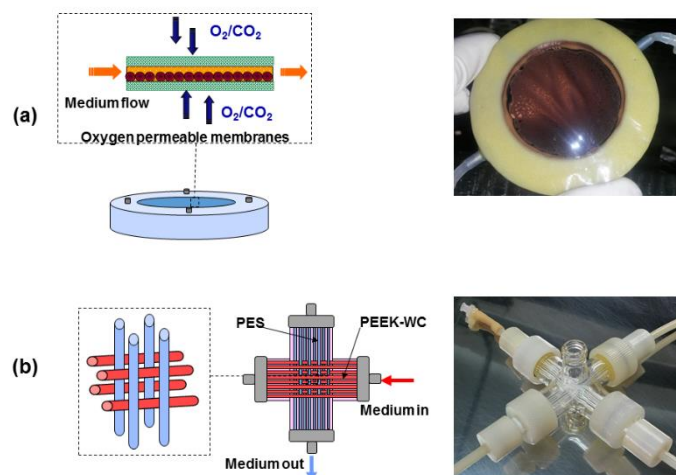


Fig.1.6. Examples of bioreactors used for *in vitro* studies. (a) Oxygen-permeable flat-membrane bioreactor (160), (b) Crossed hollow fiber membrane bioreactor (161).

Another bioreactor developed by De Bartolo et al. (161), is a HFMBR which consists of two different types of HFs arranged in a crossed configuration, mimicking the blood capillary network. Each type of HF is employed to serve a distinguished function: modified polyetheretherketone (PEEK-WC) for supplying the cells with nutrients and metabolites, and polyethersulfone (PES) for removing the catabolites. Urea, albumin and diazepam transport was mathematically modelled and experimentally evaluated. Primary human hepatocytes maintained their functionality (urea and albumin synthesis, diazepam biotransformation) for 18 days.

A different study undertook a comparative analysis of a rotating-wall gas permeable membrane system (RWMS) and a rotating-wall gas-impermeable polystyrene system (RWPS) in terms of development and cultivation of hepatocyte spheroids. Additionally, mathematical modelling permitted assessment of the oxygen, glucose and lactate transfer in these systems. Empirical work validated the mathematical prediction that RWMS made cells more viable (162).

Other researchers studied the possibility of cultivating hepatocytes into the lumen of the HF. An example of such research is the LIVER X 2000, which entrapped dispersed hepatocytes in collagen gel inoculated in the luminal compartment in their HFMBR (163). Although the system sustained hepatic functionality, it could only accommodate a limited number of hepatocytes in order to avoid nutrient limitation. The multi-bore fibre membrane bioreactor is also an instance of intraluminal cell seeding. The modified polyethersulfone fibres include seven luminal compartments displaying appropriate mechanical strength and elevated hydraulic permeance. It is possible that various cell types could be compartmentalised in every fibre with this system, enabling investigation of interaction between cells via paracrine signalling (67).

Nagaki et. al. proposed an HF module consisting of porcine hepatocytes embedded in a basement membrane matrix, Engelbreth-Holm-Swarm (EHS) gel (164). This system was reported to elevate blood bicarbonate levels, enhance hemodynamic stability and reduce levels of ammonia and lactate in trials conducted on pigs with ischaemic liver failure. Meanwhile, HFs intended for oxygenation and internal heat exchange are the basis of the OXY-HFB bioreactor. The seeding of primary porcine liver cells on the HF walls occurred within the ECS with culture medium circulation. Over a period of 3 weeks, there was preservation of hepatic functionality as regards production of urea, albumin and lactate, glucose consumption, level of oxygen and diazepam metabolism (165).

Research has also addressed improvement of cellular adhesion and functionality through the introduction of adhesive substrates in bioreactors. For example, rat hepatocytes cultured in a galactosylated polyvinylidene difluoride HF bioreactor were found to have increased albumin production (166). Similarly, a galactosylated polyethersulfone flat membrane bioreactor used in a different study maintained the functionality of human hepatocytes (167).

One commercially available device for toxicological studies is H μ REL[®] Biochip, which is a microfluidic device made up of four biochips. Evaluation of the system using primary human hepatocytes indicated the system preserved cell viability and metabolic competency at least as high as, and sometimes higher than, the traditional static culture conditions (168). Studies into the *in vitro in vivo* correlation (IVIVC) provided by this model system have shown that this system provides better IVIVC compared to the static hepatocyte cultures specially when the hepatocytes were seeded in co-culture with non-parenchymal cells (169). The model is severely limited, however, by the fact that sample removal is difficult without disturbing the

system dynamics. Furthermore, the recirculation of the medium involves a complex set of tubing lines and reservoirs, and cells on chips form monolayers and not physiological tissue constructs (170).

A closer look at the bioreactor configurations introduced above, reveals three main challenges in development of BAL devices: (i) membranes as artificial support, (ii) cell sources, (iii) mass transfer considerations.

CHAPTER 2

Long-term Maintenance of Human Hepatocyte Microtissue Spheroids in a crossed hollow fiber membrane bioreactor

2.1 Introduction

BAL devices for supporting acute liver failure patients and drug development assays require the availability of cells capable of expressing liver-specific functions for prolonged periods of time. Since the expression of several drug metabolizing isozymes cytochrome P450 (CYP450), as well as nitrogen excretion and albumin synthesis, is usually downregulated in hepatoma or immortalized cell lines (171, 172), primary hepatocytes are the main choice for BAL devices and drug development tests. Hepatocytes from animal have an inherent limitation due to the differences in metabolic pathway between species as well as the risk of xenogenic infections. Primary hepatocytes are known to maintain most of their liver-specific functions immediately after isolation (173), but lose them throughout culture time (174). Thus, a large body of work has been devoted to maintaining viable and functional hepatocytes over longer periods of time. The current standard for long-term human hepatocyte cultures is the collagen sandwich in vitro model. This model increases the interaction between cells and the matrix, providing a 3D-like architecture (60). Even though cells in this model were reported to maintain some functional and structural polarity (175), they don't faithfully recapitulate the in vivo environment in which hepatocytes are found in multi-layered microenvironment.

An alternative approach has been proposed, namely culturing hepatocytes as spheroids, in which interactions between cells can mimic the in vivo physiological structure of the cells, including phases I and II xenobiotic metabolizing activities (176, 177). Hepatocytes spheroids obtained by self-assembly of cells have the potential to be employed for the fabrication of in vitro liver tissue through fusion process with the single spheroids acting as building blocks. Using hepatocyte spheroids, have also been shown to increase liver-specific functions in cells owing to the high degree of intercellular contacts that are crucial for communication of signals and integration of gene and metabolic patterns (178). In particular, hepatocytes grown as spheroids were shown to maintain albumin production, urea synthesis, biotransformation activity for longer times than monolayer and collagen sandwich cultures (179, 180). This is likely due to the 3D cytoarchitecture, and more realistic interactions between cells and the matrix. Methods for generating 3D aggregates include the hanging drop method (181), rotational bioreactors (182), microfabricated microwells (183), selectively-

adhesive patterned structures (184), and liquid overlay method (185). The spheroid architecture, however, represents a barrier for diffusing nutrients, oxygen, drugs, and metabolic waste, especially when they reach their critical size limit. Indeed, Curcio et al. (162) reported that spheroids, with size exceeding 200 μm , in gas-impermeable systems suffer severe oxygen limitation in the most part of its size, attaining the lowest partial pressure of 12mmHg in the core. Therefore, in many bioartificial liver device the absence of a vascular network in large spheroids may cause eventually mass transfer limitations of oxygen that lead to an impairment of cell viability and functions. Indeed, a reduced cell survival (60%) due to the insufficient oxygen supply remains an issue for spheroids even when hepatocytes establish direct cell-cell interactions in coculture with human umbilical vein endothelial cells (HUVECs), which should ensure an optimal tissue-like reorganization (186).

Scaffolds such as polyurethane foams (187) and inverted colloidal crystal scaffolds have been used to limit the size of the spheroids, but their applicability is limited by potential drug adsorption onto their surface. Microfabricated substrates like microwells (183) minimize the adsorption problem, but spheroids cultured on them detach easily during routine medium changes. Thus, various methods aimed to promote spheroid adhesion, including the conjugation of integrin-binding moieties, have been studied (188, 189). However, they typically result on cell spreading and diminished hepatocyte functions (190).

To accomplish the goal of providing hepatocyte spheroids with sufficient nutrients, including oxygen, perfusion systems were employed. These systems can effectively contribute to prolonged maintenance of 3-D liver microtissue through improved mass transfer due to fluid flow. However, spheroids grown on low-adhesion or selectively-adhesive patterned substrates in perfusion culture in which they are exposed to the momentum generated by Stokes force from fluid flow, tend to ultimately detach. Moreover, shear stress generated by fluid flow negatively impacts the hepatocyte functions (191). To protect hepatocytes from shear stress, the use of microspheres and scaffolds have been proposed. However, these systems don't address the issues of spheroid loss and drug adsorption minimization (192).

Perfused microbio reactors with increased throughput have been recently developed for hepatocyte cultures (193-195). These also have inherent mass transfer limitations regarding physiologically relevant 3D structures. To overcome this limitation, higher perfusion rates are needed, which increases also the shear stress. These systems make it also hard to control essential parameters, like the dissolved oxygen or pH (196). Among those microbio reactors are microfluidic devices. The downscaling enabled by these devices yields harder to control environments, and limits their application for long term culturing. The most useful applications of micro-technologies for such

cultures couple either microfluidic perfusion or coculture micropatterning to 12- or 24-well culture plates, respectively (197, 198). Yet, they do not allow for a physiologically relevant long-term culture of hepatocytes.

When defining a culture strategy for primary hepatocyte cultures, it does not suffice to simply provide access to different nutrients based on their consumption rates. Indeed, it is crucial to maintain their physiological concentrations stable during the culture period. Several bioreactor configurations have been developed attempting to obtain a BAL device. BAL devices that use human hepatocytes are often built on hollow fiber formats. This configuration is arguably among the best-known options for maintaining large cell populations for prolonged culture periods. The configuration of these bioreactors yields a liver-like hepatic mass through the whole culture time, providing long-term endpoint assays. The bioreactors, however, do not allow high-throughput screening nor sampling the cellular mass during the culture interval (199).

In this work a crossed hollow-fiber membrane bioreactor (HFMBR) was used. The main distinguishing feature of this bioreactor, which is based on a concept developed previously (161), is the use of two bundles of hollow fiber (HF) membranes in polyethersulfone (PES): one bundle of feeding fibers to provide oxygen, nutrients and metabolites to the cells and one bundle of removing fibers to eliminate catabolites and specific products from cell compartment. The fibers are cross-assembled in alternating manner at distance each other of 250 μm in order to establish two intraluminal compartments in which medium flows and an extraluminal compartment in which microtissue spheroids are cultured and protected from shear stress. The bioreactor chamber creates a homogeneous microenvironment in which the continuous exchange of fresh and depleted media ensures the proper balance between the amount of oxygen supply and the consumption by cells, thus preventing spheroid's hypoxia and necrosis.

The underlying hypothesis guiding this work is that perfusion cultures preserve their levels of nutrients, hormones, and endogenously secreted regulators more uniformly throughout culture time. This enables a long-term maintenance of liver-specific activities in primary human hepatocyte cultures. By producing a shear stress free microenvironment with continuous feeding, no mass transfer limitations, three-dimensional cell interactions, and fully controlled culture properties, this novel system provides a robust tool for screening tests that require long-term culturing of hepatocytes, which passed the currently available high-throughput short-term screening systems.

2.2 Materials and methods

2.2.1 Mold fabrication

The MicroTissues® 3D Petri Dish® micro-mold has 256 circular recesses (16 x 16 array) and is used to self-assemble 3D microtissues in the shape of a spheroid (Fig 2.1). The nominal dimensions of one of these recesses are; diameter: 400µm and depth: 800µm. 500 ul of 2% molten agarose was added in each of the micro-molds and left for 10 minutes to set then the agarose molds were removed and placed in 12-well plates as illustrated in Fig 2.1.

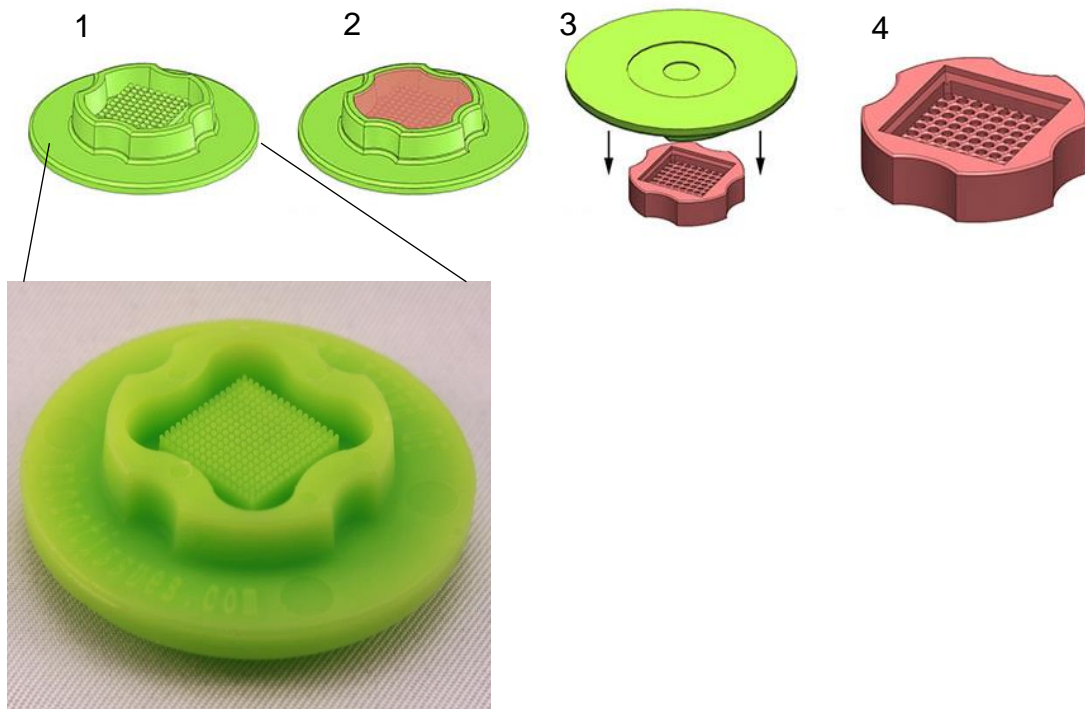


Fig. 2.1. An illustration of the various steps of agarose mold fabrication showing 1) the silicone 12-256 3D Petri Dish® cast, 2) filling the cast with molten agarose, 3) after 10 minutes the agarose mold is set and ready to be released, 4) the mold is released from the cast and is equilibrated with medium. Images taken from www.microtissues.com.

2.2.2 Bioreactor

The bioreactor consists of 180 crossed polyethersulfone (PES) HF membranes used for the medium inflow and outflow. The two fiber systems were assembled in alternating manner and potted with polyurethane adhesive (Polaris Polymers, OH, USA) within glass housing (Fig. 2.2). The fibers were potted at each end in order to establish three separate compartments: two intraluminal compartments within the inlet and outlet PES fibers, and an extraluminal compartment or shell

outside of the fibers. The intraluminal and extraluminal compartments communicate through the pores in the fiber wall.



Fig. 2.2. The crossed hollow-fiber membrane bioreactor glass housing with PES hollow-fiber membranes cross assembled into a tight mesh.

Fig. 2.3 shows the bioreactor (volume: 30 ml) that is connected to the perfusion circuit consisting of micro-peristaltic pump, gas-permeable silicone tubing and reservoir of medium. The oxygen sensor can be seen connected via an optic fiber to the data recording unit.

The oxygenated medium enters from the reservoir to the membrane bioreactor with a flow rate Q_f of 0.8 ml/min that was set on the basis of average retention time. Fresh medium was perfused in single-pass and the stream leaving the bioreactor. Q_{out} was collected as waste until approaching the steady state. When the system reached the steady state, the stream leaving the bioreactor was recycled (Q_r) in order to obtain the accumulation of products.

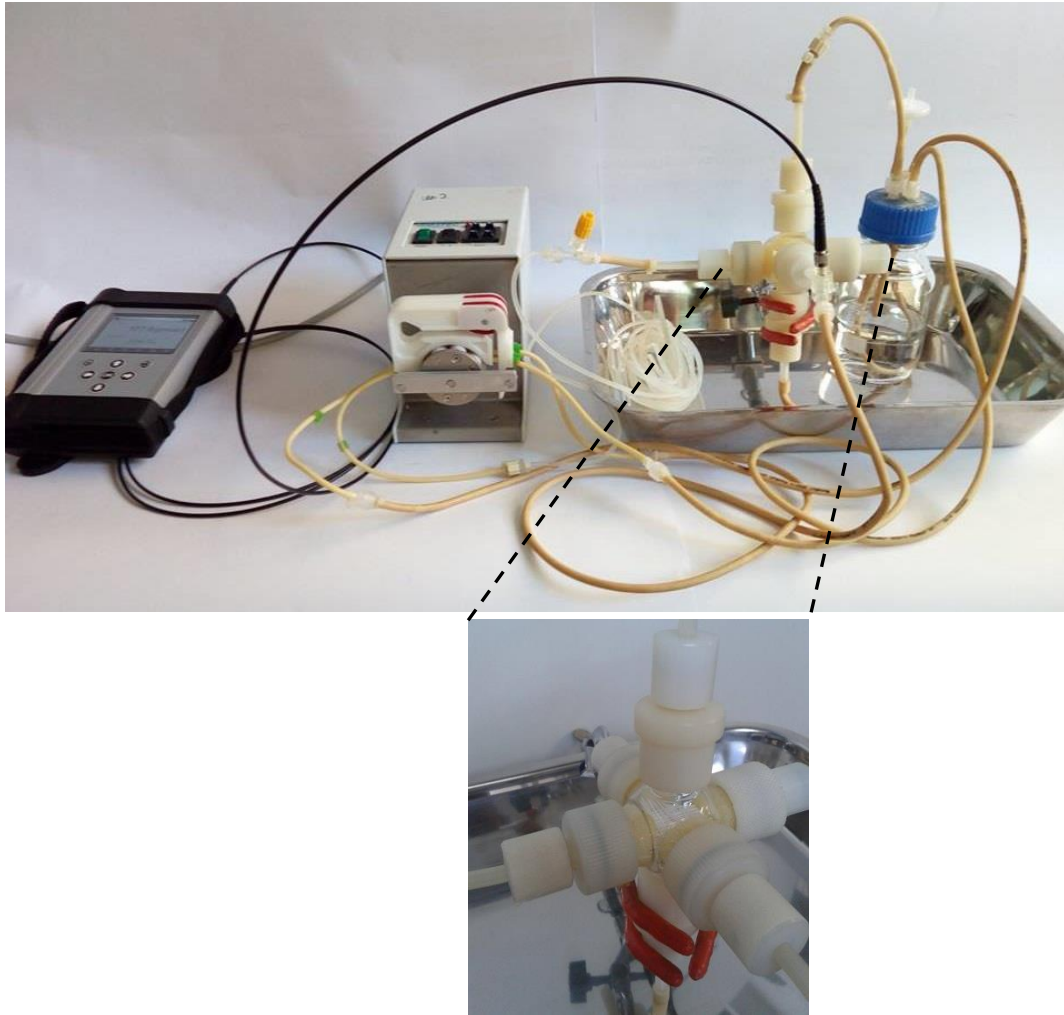


Fig.2.3. An image of the whole system showing crossed HFMBR connected to the peristaltic pump via silicone tubing and ultimately connected to the medium reservoir. The flow-through oxygen sensor is connected to the Fibox 4 meter via an optical fiber.

2.2.3 Cell Culture

Cryopreserved primary human hepatocytes (Life Technologies, California, US), isolated from human tissue were thawed in a 37° C water bath with gentle shaking. Cell suspension was slowly transferred into pre-heated 25 ml of Williams' medium E supplemented with dexamethasone 1 μ M, HEPES 15 mM, recombinant human insulin 4 μ g/ml, GlutaMAX™ 2 mM and penicillin/streptomycin (10,000 U/mL/10,000 μ g/mL) and 10% fetal bovine serum (FBS), and centrifuged at 50g at room temperature for 5 min. The viability of the hepatocytes (assessed by Trypan blue exclusion) ranged between 75 and 85%. The human hepatocytes were then seeded in 256-well agarose molds at a density of 3×10^5 cell/mold. To promote cell aggregation into spheroids, thawing medium was supplemented with 10% FBS; cells were cultured in this medium for 4-5 days.

Spheroids were then seeded into the HFMBR on the outer surface of polyethersulfone (PES) hollow fiber membranes previously sterilized and conditioned with medium containing 10% FBS. Afterwards, the FBS content in the medium was decreased gradually reaching a serum-free medium at day 17 and the experiments lasted for 25 days. The bioreactors were incubated at 37° C in 5% CO₂ with 95% relative humidity for the duration of the experiments.

2.2.4 Cell Morphology

Cell morphology in the HF membrane system was evaluated by light-inverted microscopy, Scanning Electron Microscopy (SEM, Quanta 200F, FEI, USA) and Laser Confocal Scanning Microscopy (LCSM, Fluoview FV300, Olympus Italia) analyses. Samples were examined and representative images displaying the cell features in 3D structures were obtained at different culture time.

2.2.4.1 Sample preparation for SEM

Specimens of cell cultures were prepared for (SEM) by fixation in 2.5% glutaraldehyde, pH 7.4 phosphate buffer, for 30 minutes; followed by post-fixation in 1% osmium tetroxide and by progressive dehydration in ethanol.

2.2.4.2 Staining for LCSM

Cells were fixed using 4% paraformaldehyde for 20 minutes. Afterwards, the cells were permeabilized with 0.5% triton x-100 for further 20 minutes. Hepatocyte spheroids were stained for the cytoskeleton protein actin, the tight-junction protein Vinculin and the biliary marker cytokeratin 19 (CK19). Hepatocyte spheroids were stained for the cytoskeleton protein actin, the tight-junction protein vinculin and the biliary marker cytokeratin 19 (CK19). To visualize the actin, the samples were incubated 30 min in phalloidin Alexa 488 conjugated (Molecular Probes Inc, Eugene, OR). Vinculin was visualized by using a specific mouse monoclonal antibody (Santa Cruz Biotechnology, Texas, USA) raised against human vinculin and a CyTM3-conjugated AffiniPure donkey anti-mouse IgG (Jackson ImmunoResearch Europe Ltd, Cambridge, UK). CK19 was detected by using specific goat monoclonal antibodies (Santa Cruz Biotechnology, Texas, USA) raised against human CK19 and a CyTM5-conjugated AffiniPure donkey anti-goat IgG (Jackson ImmunoResearch Europe Ltd, Cambridge, UK) were used. All primary and secondary antibodies were incubated for 2 and 1.5 hours at room temperature, respectively. Counterstaining for nuclei was performed via incubation with DAPI 0.2 µg/ml (Molecular Probes Inc, Eugene, OR) for 30 min (Salerno et al., 2011). Finally, samples were washed, mounted and viewed with CLSM (Fluoview FV300, Olympus Italia).

2.2.5 Assay of metabolic functions

2.2.5.1 Biochemical and ELISA assays

Samples of the culture medium were collected in pre-chilled tubes and stored at - 20° C until assayed. The urea concentration was assayed by the enzymatic urease method (Sentinel, Milan, Italy).

Albumin production in the samples was measured by the immunoenzymatic method (ELISA) using antibodies against human albumin. Ninety-six well plates were coated with 50µg/ml monoclonal anti-albumin antibody (Bethyl laboratories, Alabama, US) (diluted 1:100) and left at room temperature for one hour. This was followed by blocking using 10% BSA solution overnight at 4°C. Afterwards, 100 µl of cell culture supernatant was added to the wells and incubated at room temperature for one hour. Following this step, horseradish peroxidase-conjugated secondary antibody against albumin (Bethyl laboratories, Alabama, US) (diluted 1:10000) was added for one hour at room temperature. The substrate buffer containing tetramethylbenzidine and H₂O₂ (Sigma, St. Louis, MO, USA) was added for 10 min. The reaction was stopped with 0.18 N H₂SO₄. Absorbance was measured at 450 nm using a Multiskan Ex (Thermo Lab Systems).

2.2.5.2 HPLC analysis of diazepam and metabolites

Diazepam is a benzodiazepine derivative drug registered for the treatment of anxiety, insomnia, seizures, alcohol withdrawal, and muscle spasms. It is metabolized by liver cytochrome P450s to three major active metabolites; N-desmethyldiazepam or nordiazepam (NDZ), oxazepam (OX) and temazepam (TZ).

Diazepam elimination and its metabolite formation were assessed by incubating hepatocytes with 10 µg/ml diazepam. HPLC was used to assess the diazepam and metabolite concentrations in medium samples. The samples from the culture medium were alkalized with 20% of 4M NaOH, precipitated with isopropanol (1:10) and extracted with ethyl acetate (5:1) by gentle rocking for 15 min and subsequent centrifugation at 200g for 15 min at RT. Thereafter, the ethyl acetate phase was evaporated and exsiccated under vacuum condition and the pellet was dissolved in mobile phase consisting of acetonitrile /methanol /0.04%triethylamine pH 7.04 at proportion of 25/35/40, respectively. Then, samples were HPLC-analyzed using a C18-RP Purospher Star 5µm, 250x4.6 mm column, equipped with a precolumn (Merck KGaA, Darmstadt, Germany). The sample injection volume was 20µl. The mobile phase was delivered at 0.8 ml/min and the column was operated at ambient temperature. The effluents were monitored with a UV detector at 236 nm. Besides diazepam its metabolites temazepam, nordiazepam and oxazepam were detected. For all substances calibration curves were run regularly between 10 ng/ml and 10 µg/ml.

2.2.6 Dissolved oxygen concentration measurement

Dissolved oxygen concentration data was measured in HF membrane bioreactor by using a flow-through oxygen cells (Presens, Regensburg, Germany). The flow-through chemical optical oxygen sensors, based on the quenching of luminescence by oxygen, were incorporated into the culturing perfusion loop at the inlet and outlet of the bioreactor (Fig. 2.4). The sensors do not consume oxygen, are independent of the flow rate and maintain long-term stability. A fiber optic cable transmitted the optical signal between the sensor (Fig 2.5a) and the Fibox 4 portable oxygen meter (Fig 2.5b) (PreSens GmbH, Germany); the latter was connected to a PC at the end of the experiment to export the stored data in excel files which were then elaborated. The oxygen probes detected oxygen concentration with resolution of $\pm 0.4\%$ O₂ at 20.9% O₂ and a response time <30s. The membrane bioreactor containing cells was cultured in a CB 150 CO₂/O₂ incubator (BINDER, Germany). Oxygen consumption rate was estimated directly from the oxygen concentration in the inlet and outlet streams according to the steady state mass balance about the bioreactor, as follows:

$$-r = \frac{Q(C_{in} - C_{out})}{V}$$

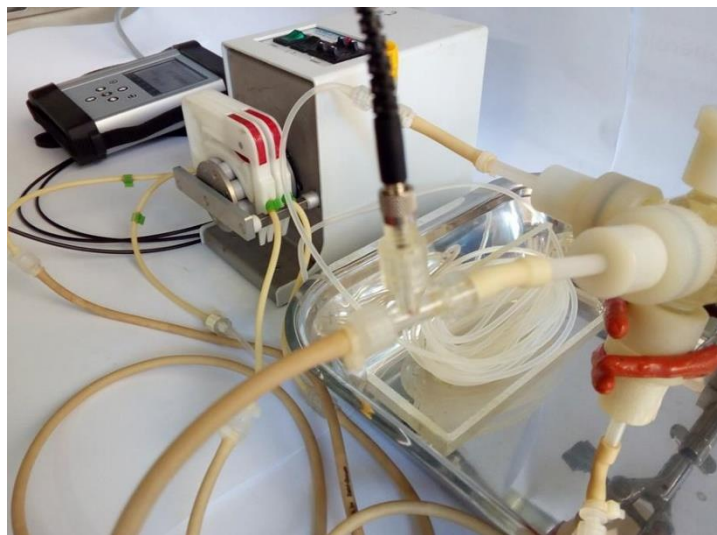


Fig. 2.4. An image of the flow-through oxygen sensor at the outlet of the bioreactor connected via optical fiber to the Fibox 4 oxygen meter.

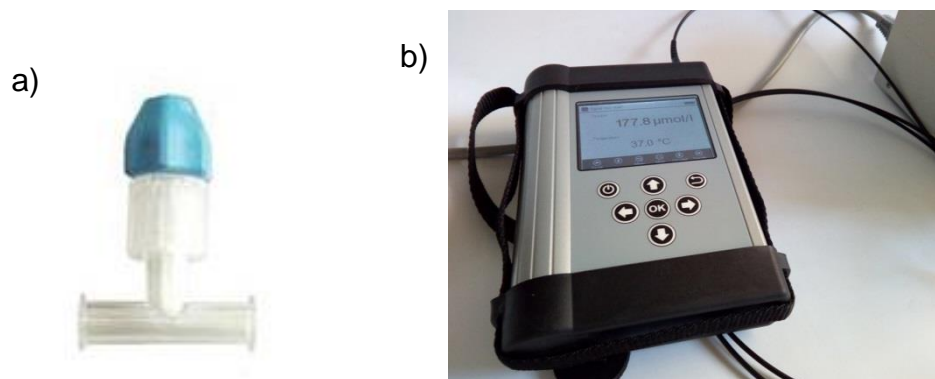


Fig. 2.5. Oxygen measurement system, a) Single-use Flow-through cell O₂, b) Fibox 4 portable oxygen meter.

2.3 Results

Evaluation of culture conditions that better granted specific hepatocyte cellular functions, using previously established biochemical assays, was carried out. Albumin secretion and urea secretion by hepatocytes are indicators for long term functional performance of hepatic cultures, whereas the activity of phase I (such as CYP 450) mirrors the capacity of hepatocytes to metabolize xenobiotics.

To study the beneficial effect of perfusion on the viability and metabolic function of hepatocyte spheroids, experiments were carried out in perfusion bioreactors to examine the possibility of sustaining the hepatocyte spheroids functional and viable for long term experiments. To this end, hepatocyte spheroids were cultured in PES-PES HFMBR with 180 hollow fibers with a flow rate of 0.8 ml/min. Samples were collected and metabolic functions, in terms of urea, albumin synthesis and diazepam metabolism, were investigated. Moreover, the morphological features of the spheroids were examined using light, scanning electron and confocal laser microscopes.

2.3.1 Morphological evaluation

Light microscope images of hepatocyte spheroids prior to seeding into bioreactors showed tightly packed cells that exhibit smooth undulating surfaces in which individual cells are indistinguishable (Fig 2.6a). As shown in Fig. 2.6b, the average diameter of the spheroids at the time of seeding is approximately 200 µm.

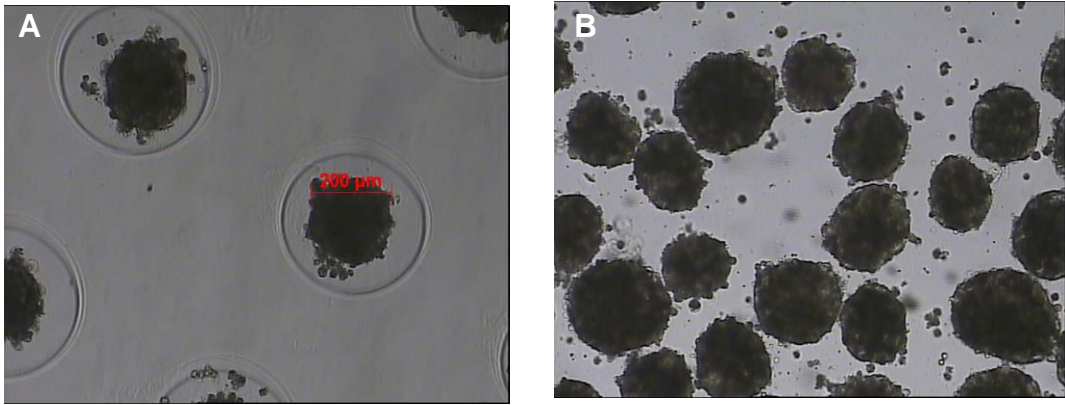


Fig. 2.6. Light microscope images of human hepatocyte spheroids at day 5, a) showing spheroids inside the agarose mold with an average diameter of 200 μm , b) showing multiple spheroids once released from the agarose molds.

After seeding in the extracapillary space of the bioreactor, spheroids barely attached to the surface of the PES fibers without alterations of their defined structure (Fig 2.7A). Spheroids tended to contract with culture time forming more compact structures with a decreased diameter due to a more tightly cell-cell interaction. Additionally, adjacent multicellular spheroids fused giving rise to larger microstructures around the fibers (Fig.2.7B).

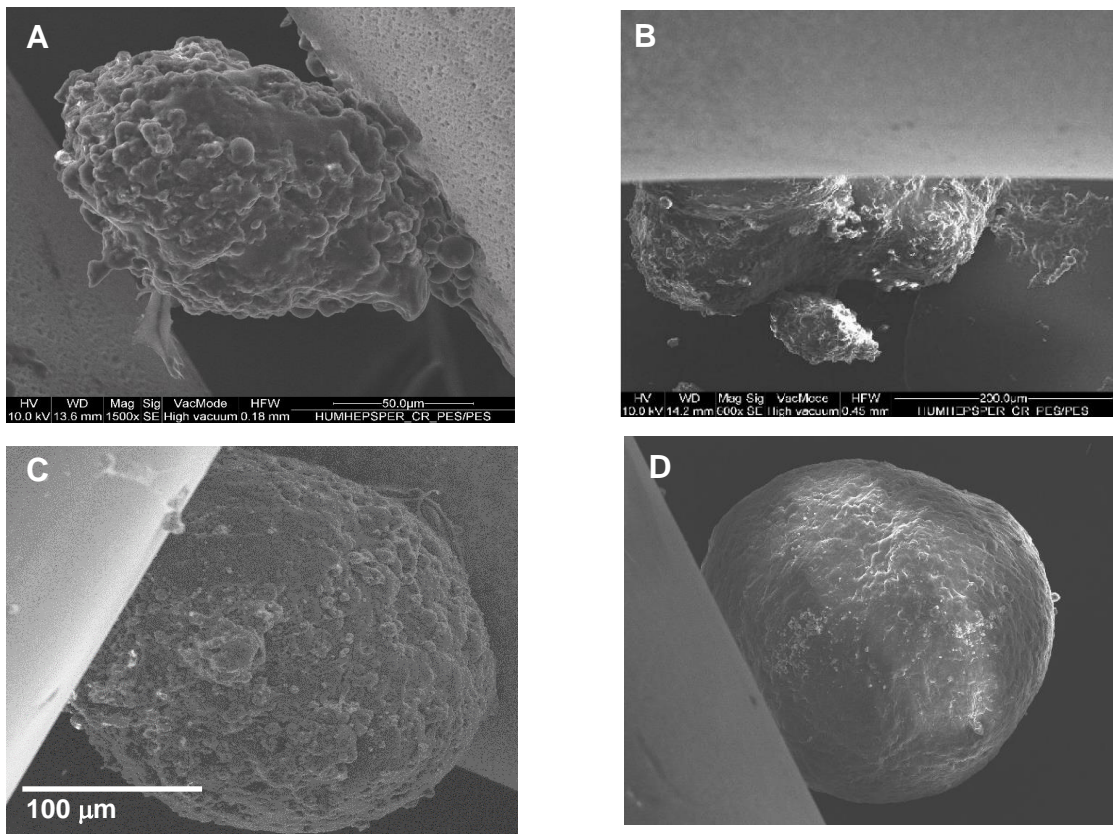


Fig. 2.7 Scanning electron microscope images of human hepatocyte spheroids cultured on crossed PES/PES hollow-fiber membranes in batch bioreactor at day 16. A and B are different magnifications of two different spheroids.

Interestingly, the fused spheroids underwent to compaction process forming liver-like tissue (Fig. 2.7C-D). Light microscope images clearly show this fusion process as it occurs over time (Fig. 2.8).

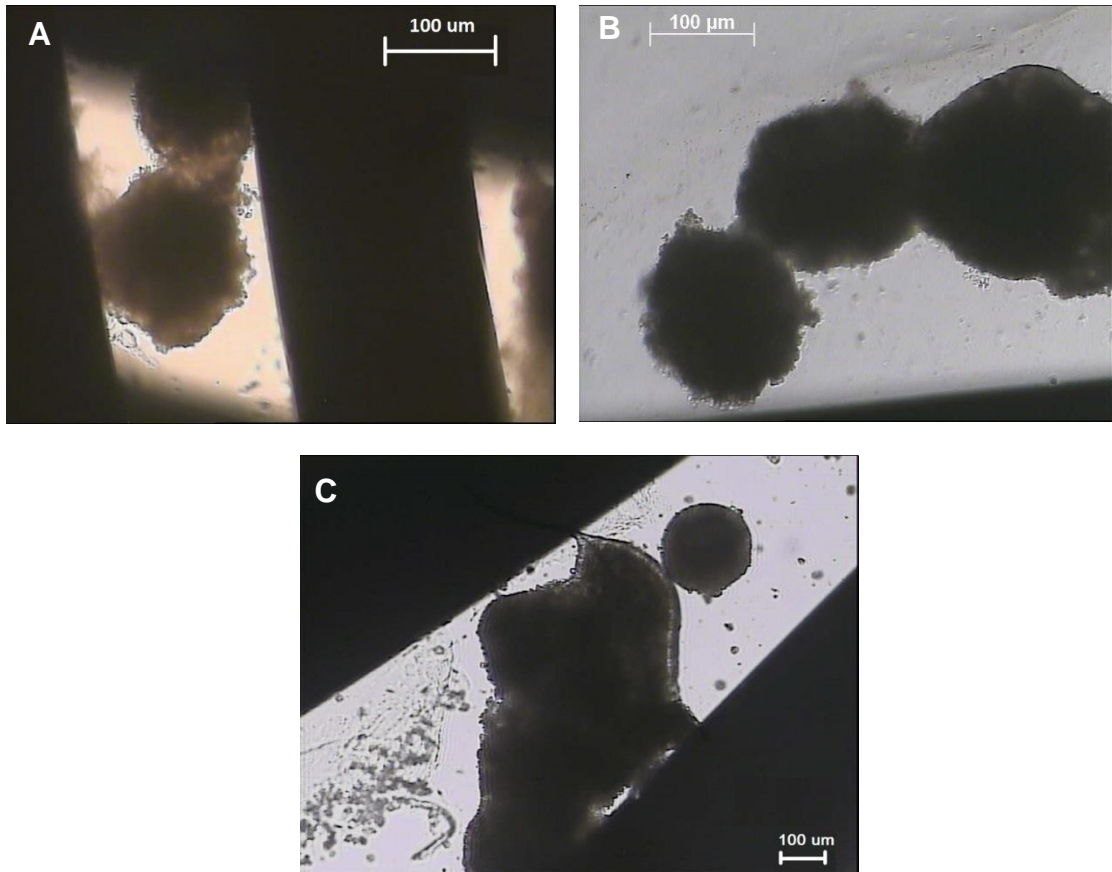


Fig. 2.8. Light microscope images showing fusing human hepatocyte spheroids seeded on PES hollow-fiber membranes a) at day 12, b) at day 15 and c) at day 22.

Immunohistochemical stainings showed multiple fused spheroids with an increased diameter of 400 µm as result of a self-organization process that makes them more compact (Fig. 2.9a-b). They establish very tight intercellular contacts originating a bigger structure with defined boundaries. Within the microtissue spheroids structure, the maintenance of a defined liver like cytoarchitecture was assessed by localization for actin (green) which was arranged according to a distribution in microfilaments, while the wide staining for vinculin (red) showed the expression of the protein at the sites of cell–cell contact as a proof of the tight junction connections between cells (Fig 2.9c-d). Hepatic spheroids within the bioreactor established and kept throughout the cultivation time high degree of cell–cell interactions and therefore their integrity and defined structure was maintained.

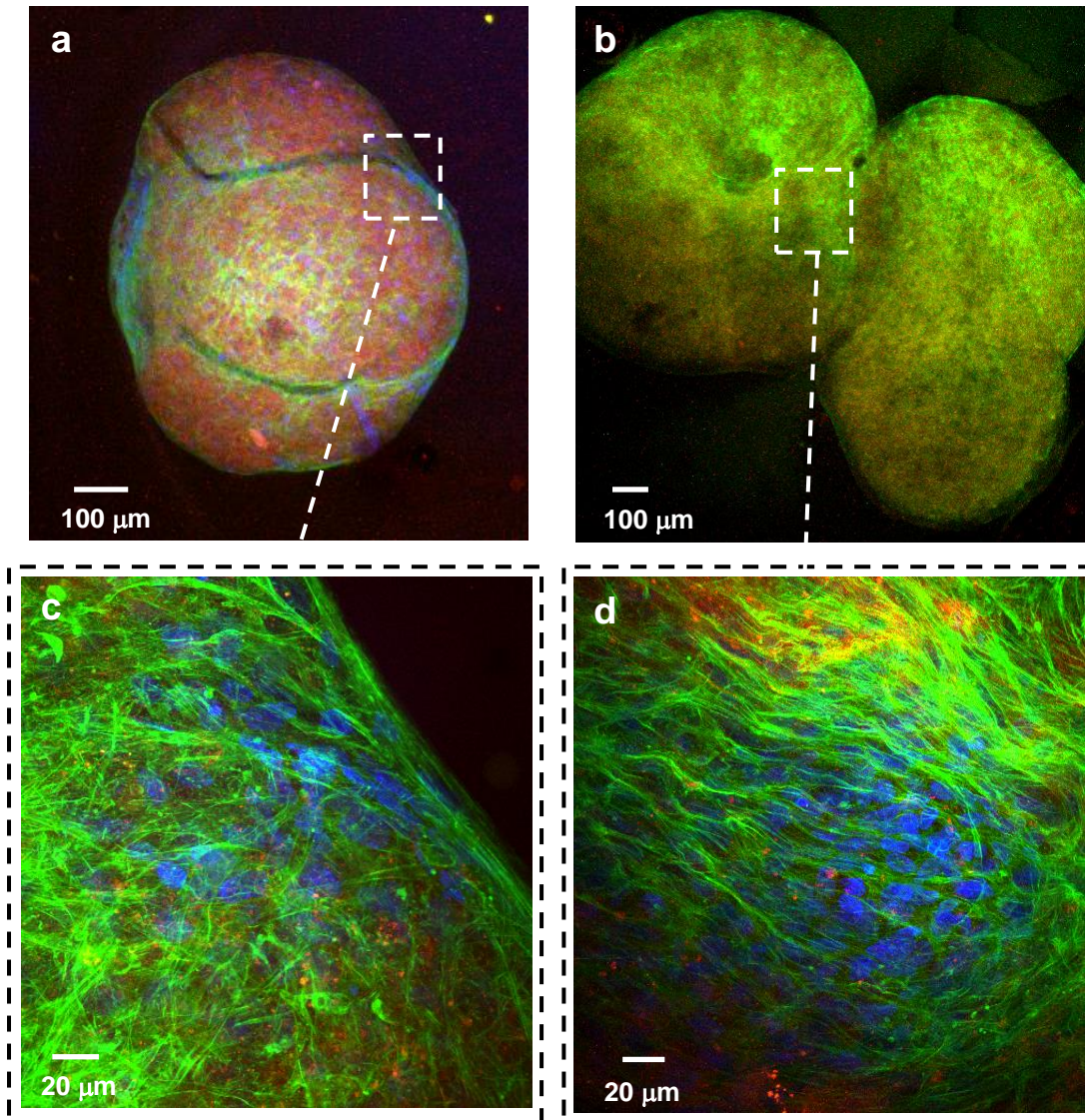


Fig. 2.9. CLSM images of the microtissues formed by the fusion of multiple spheroids (a-b) at day 18 of culture into the bioreactor. Hepatocytes were immunostained for actin (green), vinculin (red) and nuclei (blue). Magnified images of the boundary (c) and section (d) of the fused spheroids showing the distribution of actin, vinculin and nuclei.

To further investigate the creation of a liver-like structure in the new formed microtissue was also assessed the staining for CK19 as marker for cholangiocytes. CK19 positive immunolocalization indicates the presence of bile epithelial cells and the formation of bile canaliculi (Fig.2.10).

A self-assembled multicellular hepatic organization is further demonstrated by confocal images along the Z-stack. In Fig 2.11 it is possible to evaluate the hepatocytes disposition from the inner part of the spheroids towards the external one. As evidenced by the blue staining for nuclei in the core of the microtissue, cell necrosis did not occur since any picnotic nuclei are visible.

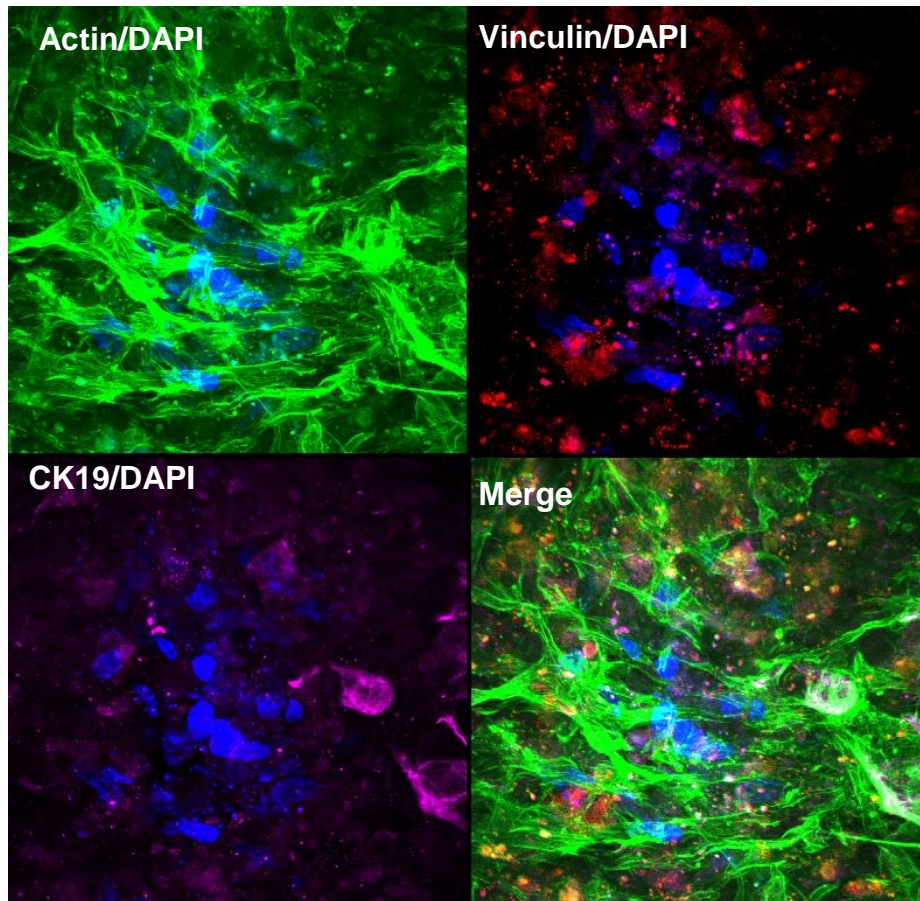


Fig. 2.10. Confocal Laser Scanning images of the human hepatocyte microtissue spheroids at day 18 of culture into the bioreactor. Hepatocytes were immunostained for actin (green), vinculin (red) CK19 (magenta) and nuclei (blue).

This result corroborated the maintenance of an adequate oxygen supply in the constructs. Hepatocytes self-assembling according to bigger structure is highlighted by the structural organization of actin. In the inner part of the spheroids actin appears in a defined distribution along the cell boundaries; moving from the inner to the outer region of the 3D construct, these boundaries are lost and the actin has rearranged according to a fasciculate orientation due to the cell spreading that occurs at the surface of the microtissues.

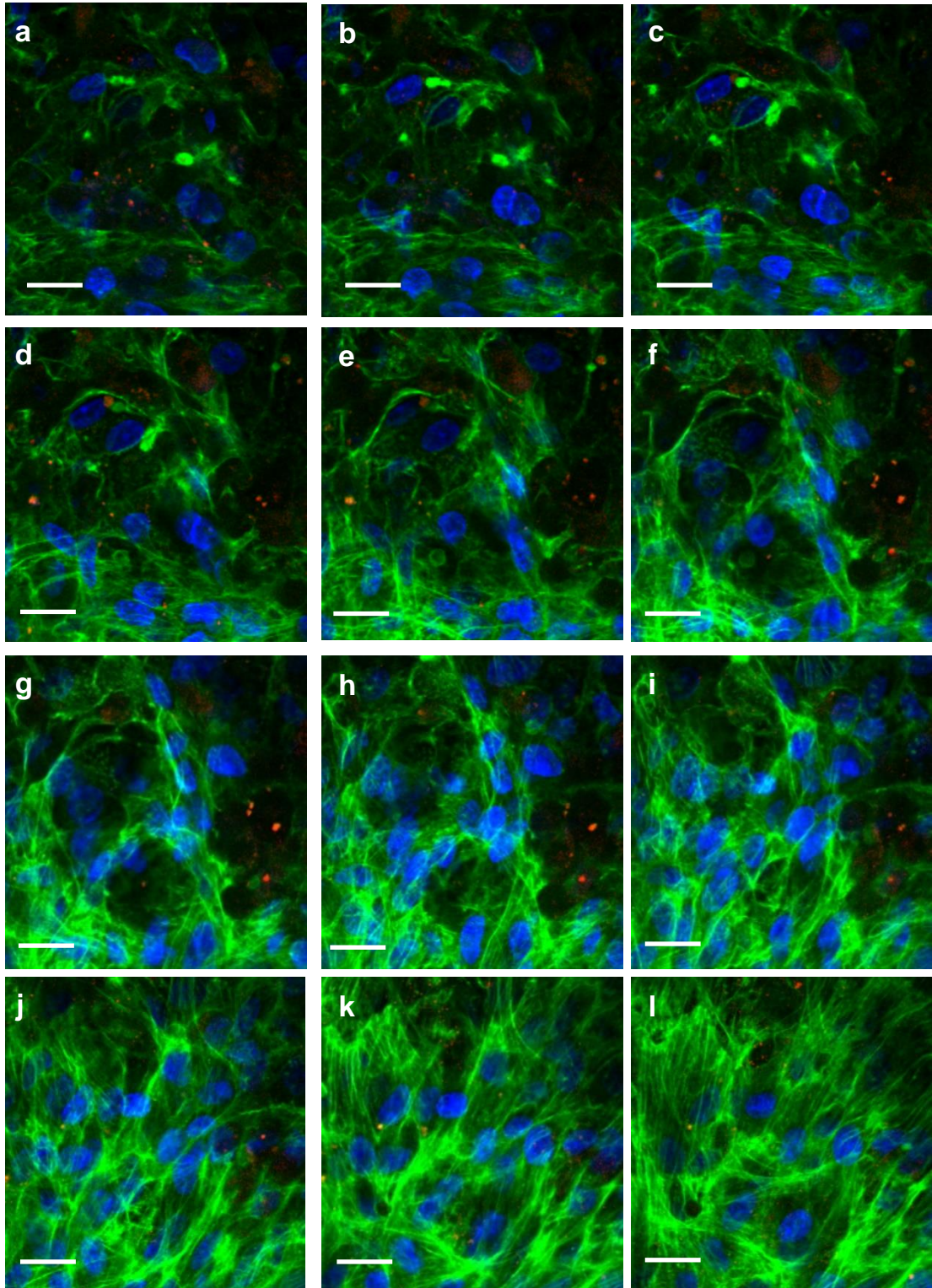


Fig 2.11 Figure 5 CLSM's images of human hepatocyte microtissue spheroids at day 18 of culture into the bioreactor. Hepatocytes were immunostained for actin (green), vinculin (red) and nuclei (blue). a-l) Progressive images of the distribution in the Z-axis of actin, vinculin and nuclei in spheroids. Z-stacks were acquired from the inner part (a) of spheroid to the external one (l).

2.3.2 Hepatocyte-specific functions

Due to the optimized culture conditions within the continuous bioreactor, the metabolic functions represented by urea, albumin synthesis and diazepam metabolism were maintained throughout the experiment lasting for up to 25 days post-thawing. Values have been normalized to the number of seeded hepatocyte cells at the beginning of the experiment.

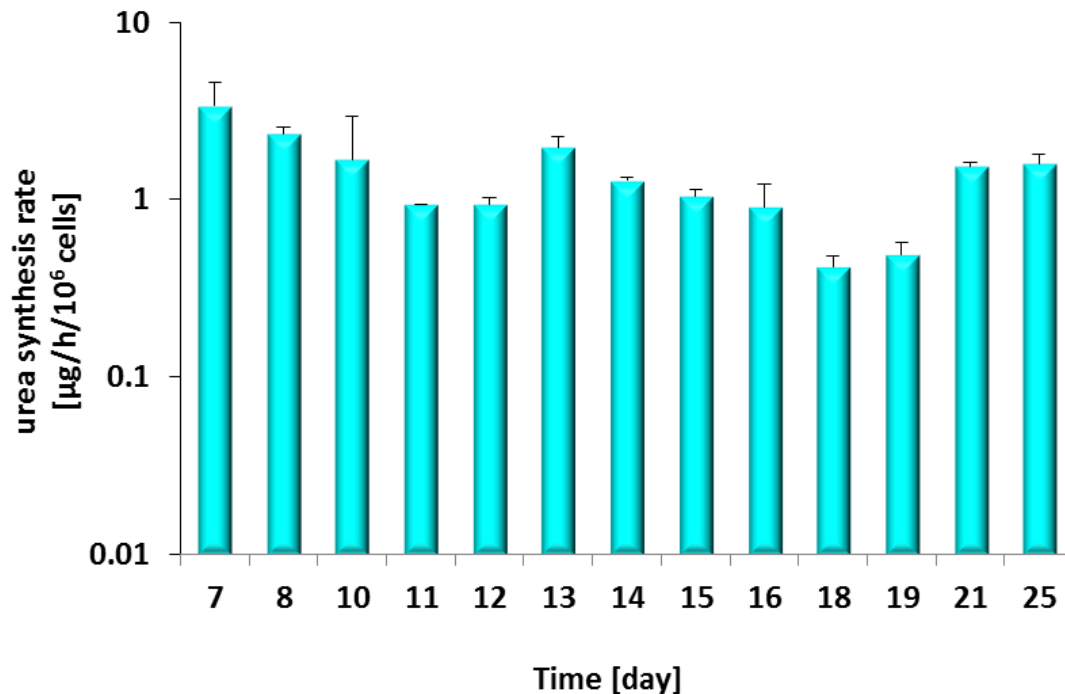


Fig. 2.12. Urea synthesis rate of human hepatocyte spheroids cultured in PES/PES crossed HFMBR. The values are the mean of three experiments \pm standard deviation.

Urea synthesis rates were high at the beginning of the experiment reaching values of approximately $4.38 \mu\text{g}/\text{h} \cdot \text{million cells}$. The synthesis rate then decreased with the decrease of serum concentration in the medium. Upon complete removal of serum from medium at day 17, urea synthesis rate dropped to a value of approximately $0.5 \mu\text{g}/\text{h} \cdot \text{million cells}$ at day 18. Interestingly, after a period of cell's adaptation to the new culture condition, urea synthesis rate increased again up to value of approximately $1.6 \mu\text{g}/\text{h} \cdot \text{million cells}$ (Fig. 2.12), this new trend was observed from day 21 until the end of the experiment.

Albumin synthesis was detected for the entire cultivation period of 25 days, indicating that, within the bioreactor the well-structured spheroid micro tissues were retaining their specific function. Quite soon after the seeding in the bioreactor the albumin was present in the samples at a very high value of around $49 \text{ ng}/\text{h} \cdot \text{million cells}$ owing to the presence of serum, which was gradually

depleted in the culture time. Afterwards, the albumin synthesis rate was almost stable until day 14 with values of approximately 27 ng/h*million cells. A new steady state was then reached between day 15 and day 23, in which the albumin synthesis rate was approximately 22 ng/h*million cells. Only at the end of the culture was registered a slight decrease in the albumin secretion reaching a value of approximately 13 ng/h*million cells (Fig. 2.13).

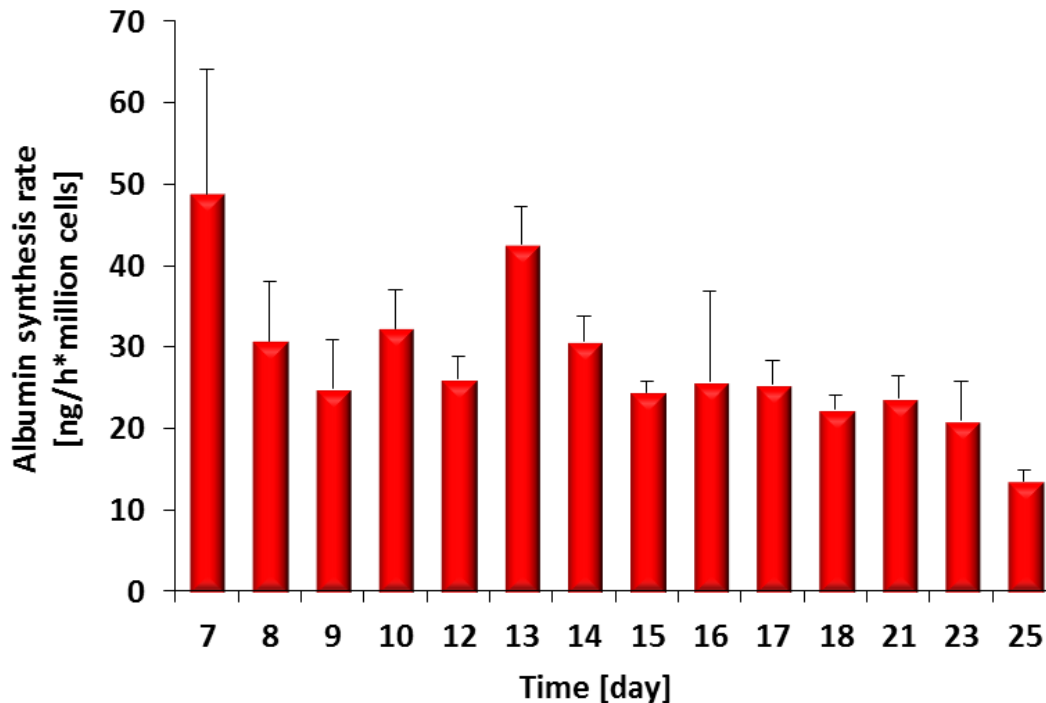


Fig. 2.13. Albumin synthesis rates of human hepatocyte spheroids cultured in PES/PES crossed HFMBR. The values are the mean of three experiments \pm standard deviation.

The assessment of the phase I enzymes' functionality (such as CYP 450) mirrors the capacity of hepatocytes to metabolize xenobiotics. CYP 450 activity of hepatocytes spheroids was evaluated through the detection of diazepam metabolites formed after the drug administration in the cell culture media. Drug metabolism is an important function of hepatocytes that is usually lost after few days in culture, since the involved enzymes are quite sensitive to the surrounding microenvironment. Thanks to the optimal culture conditions within the bioreactor, the diazepam was metabolized and the main metabolites of phase I were detected throughout the experiment. For each analyzed time point, HPLC investigation evidenced that diazepam was eliminated with an average rate of 4 ng/h/10⁶ cell throughout the time (Fig. 14a). Accordingly, the metabolites of diazepam were detected with nordiazepam (NDZ) and temazepam (TMZ) being produced at the highest rate while oxazepam (OXZ) was detected at lower rate compared to the other two metabolites (Fig. 14b). Noteworthy, biotransformation

capacity was retained even in serum free condition, highlighting the efficient performance of the bioreactor to preserve very sensitive hepatic functions.

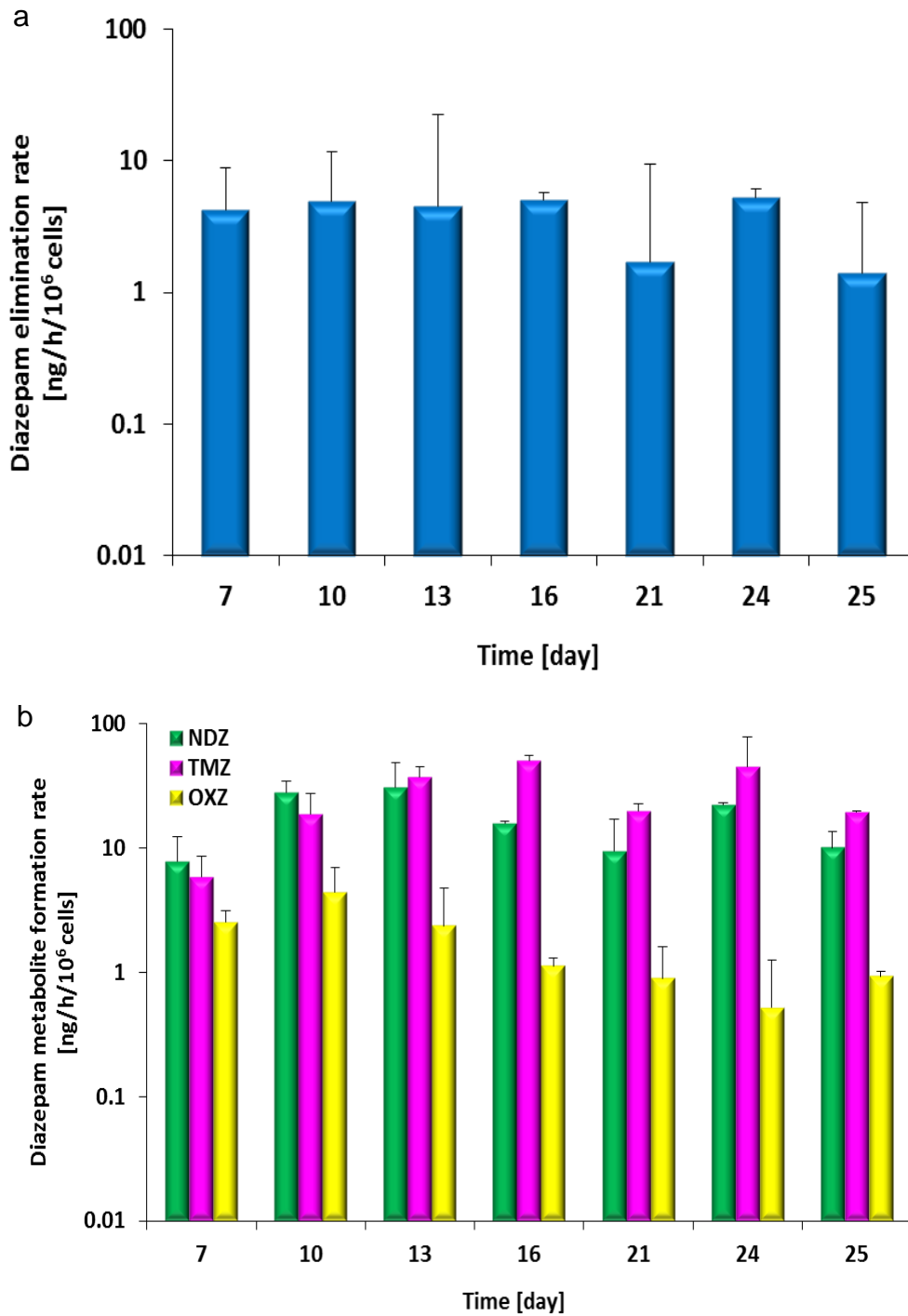


Fig. 2.14 Biotransformation functions of microtissue spheroids expressed in terms of diazepam metabolism. a) Diazepam elimination rate and b) nordiazepam (NDZ) and temazepam (TMZ) and oxazepam (OXZ) formation rates. The values are the mean of three experiments \pm standard deviation.

2.3.3 Oxygen uptake

The ability of the membrane bioreactor to provide a suitable microenvironment, in which oxygen diffusion limitation is avoided, was investigated by a constant quantification of the cellular oxygen uptake and consumption. Indeed, the presence of oxygen sensors at the inlet and outlet of the bioreactor allowed an online monitoring of the oxygen measurements. The oxygen concentration was stable at 210 $\mu\text{mol/L}$ in the inlet medium and fluctuated between 175 and 195 $\mu\text{mol/L}$ in the outlet medium due to cell uptake (Fig. 2.15a). The oxygen consumption was registered for 25 days with values ranging from 800 to 1500 nmol/h (Fig 2.15b) until the end of the culture. Hepatocytes have elevated oxygen demands, the possibility to maintain a proper oxygen consumption within the chamber device is fundamental to afford the highly energetic need of hepatic cells, which in turn are able to keep their specific metabolic activities.

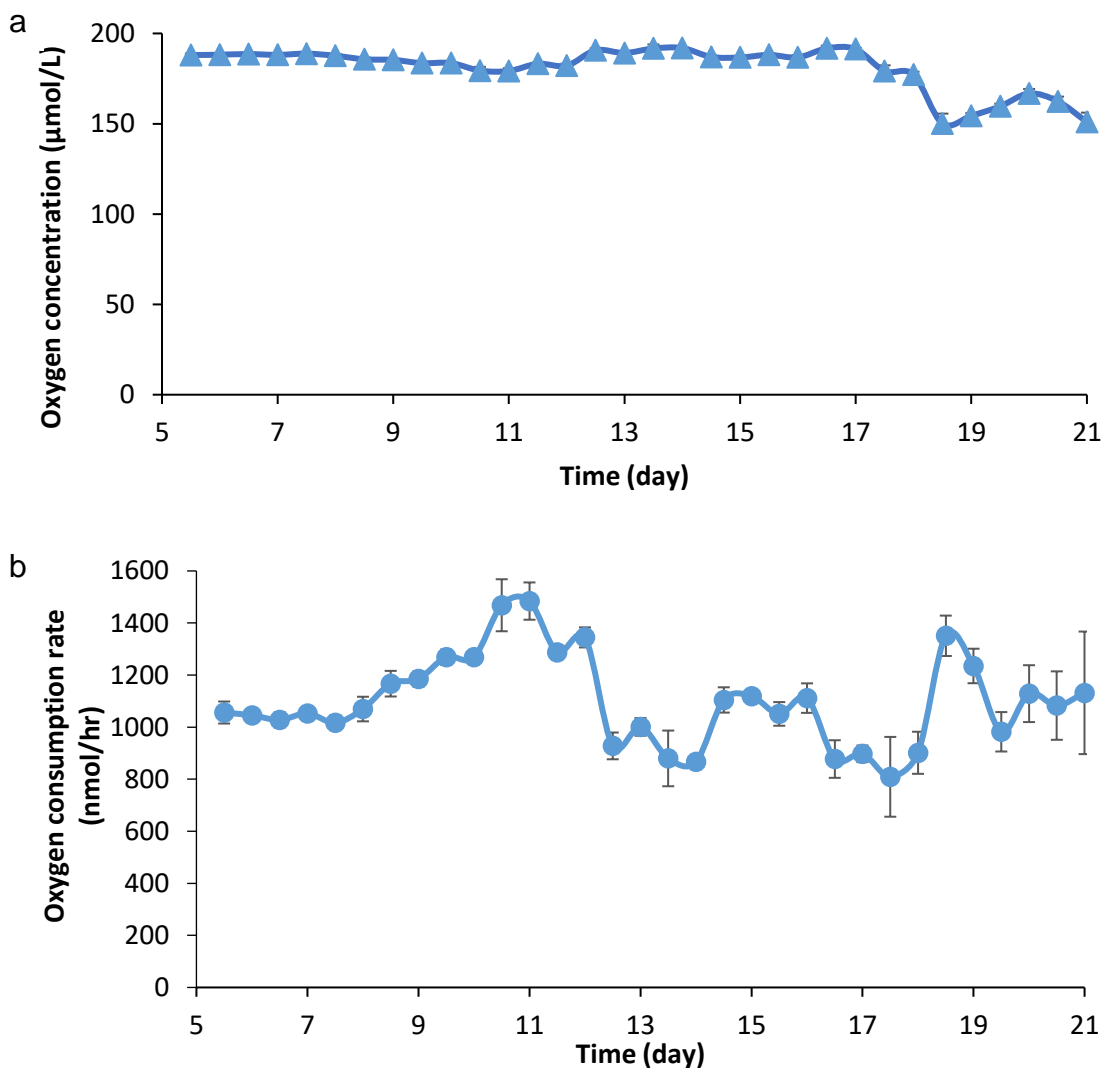


Fig. 2.15. a) Oxygen concentration in the outlet stream of the bioreactor; b) oxygen consumption rates of human hepatocytes microtissue spheroids cultured into the bioreactor. The values are the mean of three experiments \pm standard deviation.

2.4 Discussion

The aim of this work was to develop metabolically active human liver microtissue spheroids by using an optimized crossed HF membrane bioreactor that was optimized in order to ensure a uniform microenvironment and adequate oxygenation. It is well known that the absence of a vascular network in large spheroids may eventually cause oxygen transfer limitations leading to impairment of cell viability and functions. In order to provide hepatocyte spheroids with sufficient amount of oxygen and other nutrients, and to efficiently remove catabolites and cellular products, we used a crossed hollow fiber membrane bioreactor for the spheroid culture. The bioreactor thanks to the selective mass transfer properties of the membranes and the fluid dynamics conditions creates a well-controlled cellular environment at the molecular level allowing the continuous feeding of nutrients and washing-out of catabolites and cellular specific products, which are necessary for the homeostasis of the system. Uniform levels of nutrients, hormones, and endogenously secreted regulators throughout culture time are important for cells to perform their specific functions.

An important scientific achievement in this study was the development of an alternative in vitro human liver platform that can be used for both toxicological and pharmacological studies. The choice of a 3D system was based on the fact that hepatocytes retain both high viability and differentiated liver functions when cultured as 3D aggregates. This was shown by Leite *et al.* (196) who successfully cultured rat hepatocyte spheroids, embedded in alginate beads, in a stirred-tank bioreactor for 3 weeks. Aggregates could be cultured for a prolonged period with differentiated cell morphology and functions. For spheroids ranging between 65 and 81 microns this system offered a tight control of critical environmental variable as the oxygen transport at physiological level. For a more realistic liver tissue organoids, spheroids of bigger size are preferred. This aspect raises some issues about nutrients and oxygen transport within the spheroid, which could lead to cell death in the core of the spheroid. The problem is even more relevant if we consider that within a bioreactor, spheroids are naturally led to form bigger construct as the result of the fusion of different spheroids. The advantage of our system over previously described in vitro models is that it conjugates several characteristics that are important for functional hepatocytes culture and for culture practicability in general. The cells were cultured in a fully controlled environment bioreactor where sampling throughout the whole culture time was made possible. In addition, primary human hepatocytes were used which better reflects the in vivo situation in humans when compared to rat hepatocytes or cell lines (200). One major advantage of the hollow-fiber membrane bioreactor used in this study is that it allows spheroids to interact fusing into larger microtissues

that better resembles the in vivo environment as confirmed by light microscope and confocal laser microscope images.

Liver specific functions such as albumin and urea secretion are important indicators when evaluating hepatocyte functionality. Albumin secretion is a general marker of protein synthesis in hepatocytes as it is synthesized almost entirely by the liver. It is a source of amino acids for various tissues. Decreased albumin level usually indicates an impaired synthetic protein capacity by the liver (201). Likewise, the conversion of ammonia to urea is a vital liver function. The ammonia produced through amino acid deamination can be detoxified by the combination of ammonia with CO₂ to form urea in the liver (202).

Urea synthesis was maintained for the whole period of the experiment. The decrease exhibited during the first 17 days can be explained by the fact that serum concentration in the medium has been decreased from 10% at the beginning of the experiment to reach 0% at day 17. Serum is a complex component of blood that contains various amino acids and ammonia that can be converted by hepatocytes into urea. A similar behavior was observed in albumin synthesis, possibly due to the positive effect that hepatocyte growth factor (HGF), found in the serum, on albumin synthesis as reported previously (203).

Biotransformation of xenobiotics by cultured hepatocytes can be assessed in terms of activity using specific substrates. Diazepam, an anti-anxiety drug, was used as a model drug in our studies. All 3 major metabolites, namely NDZ, TMZ and OXZ, were detected to varying degrees during the experiment. One parameter further controlled in the bioreactor is the dissolved oxygen, a key factor on hepatocyte cell long-term cultures. Therefore, an oxygenated environment may be the reason why better results are observed in the bioreactor. It has been shown that the absence of oxygen in culture can lead to a cellular metabolic state of sensitive or even rapid hypoxic injury, due to the depletion of dissolved oxygen; regular culture conditions do not allow this control as a bioreactor would (149).

Hepatocyte spheroids had a mean diameter of 200 µm on seeding into the bioreactor as shown in light microscope images. Spheroids shrink over time, however, single spheroids fuse forming larger microtissues. In order to avoid any possible oxygen limitation, the flow rate may be increased to allow for better oxygenation of the medium; moreover, oxygen concentration in the incubator can be increased.

The hollow fiber membranes played a key role in the mass transfer of nutrients including oxygen to the microtissues being highly permeable (hydraulic permeance of 15.2 L/m²h mbar). This property boosted the fusion of spheroids in microtissue around and between the fibers, which served as capillary system. Another important and encouraging aspect of our membrane bioreactors lies in the physico-chemical properties of PES membrane surface that contribute to keep defined construct boundaries with small point of adhesion. In the spheroids

cells are approached each other closely and their interaction is stronger than that with the membrane surface. Therefore, the spheroids maintained their aggregation condition without any spreading phenomenon and subsequent alteration of their shape as result of limited interaction with the membrane surface.

By more closely resembling the *in vivo* environment, due to its three-dimensionality and cell–cell interaction, this model supported long-term viability and the maintenance of several differentiated hepatocytes properties, including xenobiotic metabolism, for up to 25 days. In contrast, traditional monolayer cultures lost these properties after 3–7 days in culture. Therefore, this system offers a much-improved alternative culture system for predictive *in vitro* studies, to study mechanisms of CYP isoforms and to perform metabolic stability studies. It is also a new method for testing drug interaction under 3D conditions, because it has the advantage of offering a better *in vitro*–*in vivo* correlation.

2.5 Conclusion

In this study, a 3D liver micro-tissue model was developed using human hepatocyte spheroids that were realized by seeding single cells into agarose molds of defined diameter achieving spheroids of homogenous sizes. The spheroids were then seeded into the crossed hollow-fiber membrane bioreactor (HFMBR) made up entirely of PES hollow fibers, where the spheroids were successfully maintained viable and functional for 25 days, as shown through urea, albumin synthesis and diazepam metabolism analyses. Furthermore, the inertness of the PES hollow-fiber membranes allowed for weak adhesion of the spheroids, which in turn allowed them to move and fuse together forming larger microtissue-like structures that more closely resembles the *in vivo* environment. The HFMBR provides a well-controlled microenvironment through continuously feeding nutrients, most important of which is oxygen, and removing catabolites. This was confirmed via dissolved-oxygen monitoring in both inlet and outlet of the bioreactor. This lab-scale bioreactor employing human hepatocyte spheroids thus can be used to study toxicity of pharmaceuticals that need long-term cultures. Moreover, it can be scaled up to clinical scale to be used for liver-patients support.

CHAPTER 3

3D organotypic liver organoid formation in hollow fiber membrane systems

3.1 Introduction

In vitro strategies to create a liver model closer to the *in vivo* situation are still challenging and sought after for clinical application and pharmacological research. Biofabrication approaches can recapitulate the three-dimensional microenvironments of tissues promoting cell–cell and cell–matrix interactions. The liver tissue assumes structurally complex and well-organized architecture where the parenchymal component, hepatocytes, performs a wide spectrum of liver specific functions and is in tight contact with non-parenchymal cells. Drug-induced liver injury, caused by chemicals and their metabolites, cannot be fully predicted by current animal models which provide limited physiologically relevant information due to the species-specific gene expression variation between animal and humans: resulting in the introduction of many hepatotoxic drugs into clinical trials (204). Human-based *in vitro* models comprising microsomes, cell lines, primary hepatocytes, and liver slices (11, 13, 205) provide additional information to the existing animal models. However, they can be limited by poor stability, and, with the exception of precision-cut liver slices, lack the hierarchy and structural components of liver. Even though monolayer cultures of primary hepatocytes, which are the most commonly used *in vitro* format for toxicity assessment, provide a suitable model for initial assessment, they cannot fully predict hepatotoxicity of drugs because of the lack of a 3D organization, organotypic cell–cell interactions via contact or paracrine effects. Furthermore, primary hepatocytes are known to lose their differentiated phenotype and viability rapidly in a traditional cell culture system because they are isolated from their native microenvironment (206).

Recent investigations into the molecular pathology of liver injury reveal the important role of non-parenchymal cells, such as resident liver immune cells, stellate cells, and endothelial cells, in the pathologic lesion through their interaction with the liver parenchyma (207). Clearly, there remains a need to develop more physiologically-relevant, sophisticated long-term culture systems for identifying pathways involved in drug-induced toxicity, allowing for *in vitro-in vivo* extrapolation (208). An *in vitro* human liver model must address the interactions between the major human liver cell types in order to be predictive of *in vivo* condition.

While traditionally collectively referred to as “other cells” when discussing hepatocytes, non-parenchymal cells actively contribute to the support of

hepatocyte viability and functionality (209). Sinusoidal endothelial cells (SEC) actively secrete cytokines and some extracellular matrix (ECM) components (210, 211); furthermore, they also contribute to both phase I and phase II metabolism and are the target of some hepatotoxic chemicals (212, 213). Mature hepatic stellate cells (HSCs) produce ECM components such as collagen, elastin and proteoglycans (214, 215). HSCs also synthesize and release a number of cytokines and growth factors vital for intercellular communication in normal and injured livers. Several research groups have shown that the use of stellate and sinusoidal cells is indeed useful for maintaining hepatocyte functionality in vitro (216-218). Co-culture systems have been developed by using a mixture of primary NPC or cell line in co-culture with hepatocytes for the improvement of their in vitro functionality (207, 219, 220). Recent studies have shown that biohybrid membrane systems provide an appropriate microenvironment for cell adhesion, proliferation and differentiation. Indeed these advanced devices mimic specific features of in vivo environment allowing the successful achievement of in vitro engineered tissues (221, 222). Co-culture systems have been developed by using PLGA e PDMS membranes with parenchymal and non-parenchymal cells (223, 224). These studies have been performed in static conditions by using cells isolated from rats.

For the first time, the current work utilizes a designed approach based on the sequential seeding of primary human sinusoidal endothelial cells, stellate cells and hepatocytes on hollow fiber (HF) membranes of polyethersulfone (PES) in static and dynamic conditions for the development of a 3D liver system. The HF membranes allow the compartmentalization of cells in a microenvironment controlled at molecular level and the selective mass transfer of molecules to and from the cell compartment without causing shear stress. Owing to its intrinsic geometry they provide a wide surface area for the adhesion and growth of cells in a small volume. Dynamic conditions were established by using a crossed hollow fiber membrane bioreactor based on a concept developed previously [24]. This bioreactor consists of two bundles of HFs that are cross-assembled in alternating manner at distance of 250 μm each other in order to establish two intraluminal compartments in which medium flows and an extraluminal compartment in which cells are cultured in adhesion on the outer fiber surface. The feeding fibers provide nutrients and metabolites to the cells and removing fibers eliminate catabolites and specific products from cell compartment ensuring the homeostasis of cellular microenvironment.

3.2. Materials and Methods

3.2.1. Membrane systems

Polyethersulfone (PES) HF membranes (Membrana, GmbH) were used for the creation of batch system and bioreactor. The HF membranes were modified with fibronectin coating (2 $\mu\text{g}/\text{cm}^2$). The membranes were characterized in order to evaluate the wettability, MW cut-off and hydraulic permeance. The wettability of the membranes was specified by water dynamic contact angle (DCA) measurements, which were performed with a CAM 200 contact angle meter (KSV Instruments, Ltd., Helsinki, Finland). The hydraulic permeance of the membranes was evaluated by pure water flux measurements in the absence of solutes and at different transmembrane pressure (ΔP^{TM}). The hydraulic permeance L_p , was measured by the following equation:

$$L_p = \left(\frac{J_{Solvent}}{\Delta P^{TM}} \right)_{\Delta c=0}$$

This equation assumes a linear correlation between water flux and the convective driving force. The membrane bioreactor was realized according to a previous concept (161) with some modification. The device consists of two bundles of 100 PES HF membranes cross-assembled in alternating manner at distance of 250 μm and potted with polyurethane adhesive (Polaris Polymers, Avon Lake, OH, USA) within glass housing. The two fiber systems are used for the medium inflow and outflow, respectively. Two separate intraluminal compartments within the PES fibers and an extraluminal compartment or shell outside of the fibers are established. The lumen and extraluminal compartments communicate through the pores of the fiber wall. The oxygenated medium enters from the reservoir to the membrane bioreactor with a flow rate (Q_f) of 1.0 mL/min that was set on the basis of average retention time. Fresh medium was perfused in single-pass and the stream leaving the bioreactor (Q_{out}) was collected as waste until approaching the steady state. When the system reached the steady state, the stream leaving the bioreactor was recycled (Q_r) in order to obtain the accumulation of products.

The fluid dynamics of the bioreactor were optimized and characterized in terms of cumulative residence time distribution (RTD), which was investigated through the introduction of tracer (step input) at the entrance of PES fibers and recording it in time at the exit.

3.2.2. Cell Culture

3.2.2.1. Homotypic culture

Cryopreserved primary human hepatocytes (Life Technologies, California, US), isolated from human tissue were thawed in a 37° C water bath with gentle shaking. Cell suspension was slowly transferred into pre-heated 25 ml of Williams' medium E supplemented with dexamethasone 1 µM, HEPES 15 mM, recombinant human insulin 4 µg/ml, GlutaMAX™ 2 mM and penicillin/streptomycin (10,000 U/mL/10,000 µg/mL) and 10% fetal bovine serum (FBS), and centrifuged at 50g at room temperature for 5 min. The viability of the hepatocytes (assessed by Trypan blue exclusion) ranged between 80 and 90%. The human hepatocytes were then seeded at a density of 10⁵ cells/cm² on the outer surface of HF membranes previously sterilized and conditioned with medium containing 5% fetal calf serum (FCS). After 24 hours, the medium was removed and replaced with serum-free medium. Cells and controls were incubated at 37° C in 5% CO₂ with 95% relative humidity for the duration of the experiments.

3.2.2.2. Organotypic culture

Human sinusoidal endothelial cells (Sciencell, California, United States) were expanded in fibronectin-coated (2 µg/cm²) T-75 flasks in endothelial cell medium (ECM) supplemented with FBS 5%, bovine serum albumin (BSA) 10µg/ml, apotransferrin 10µg/ml, insulin 5µg/ml, endothelial growth factor (EGF) 10ng/ml, basic fibroblast growth factor (FGF-2) 2ng/ml, vascular endothelial growth factor (VEGF) 10ng/ml, insulin-like growth factor (IGF-1) 2ng/ml, hydrocortisone 1µg/ml and retinoic acid 10⁻⁷ M. Upon reaching 70-80% confluence, cells were seeded at a density of 4*10³ cells/cm² on the outer surface of HF membranes at day -2, using ECM supplemented with 5% FBS (Fig.1). After 24 hours, human stellate liver cells (Sciencell, California, US), previously expanded in poly-l-lysine-coated T-75 flasks (2 µg/cm²) in stellate cell medium (SteCM) supplemented with FBS 2%, BSA 10µg/ml, apotransferrin 10µg/ml, insulin 5µg/ml, EGF 2ng/ml, FGF-2 2ng/ml, IGF-1 2ng/ml and hydrocortisone 1µg/ml, were seeded at a density of 4*10³ cells/cm² on the outer surface of HF membranes at day -1, using a mixture of ECM and SteCM supplemented with 3.5 % FBS.

After 24 hours (day 0), human primary hepatocytes were over seeded at a density of 10⁵ cells/cm² into the co-culture membrane system (Fig. 3.1) in a medium composed of a mixture of the 3 cell culture media. FBS was used at a concentration of 5% for the first day of culture and then it was removed afterwards. Co-culture batch systems were incubated at 37°C in 5% CO₂; 20% O₂ atmosphere (v/v) with 95% relative humidity for the first 7 days and then O₂ was increased to 25% until day 9 then further increased to 30% for the rest of the experiment. Medium was changed daily and samples were taken for analysis.

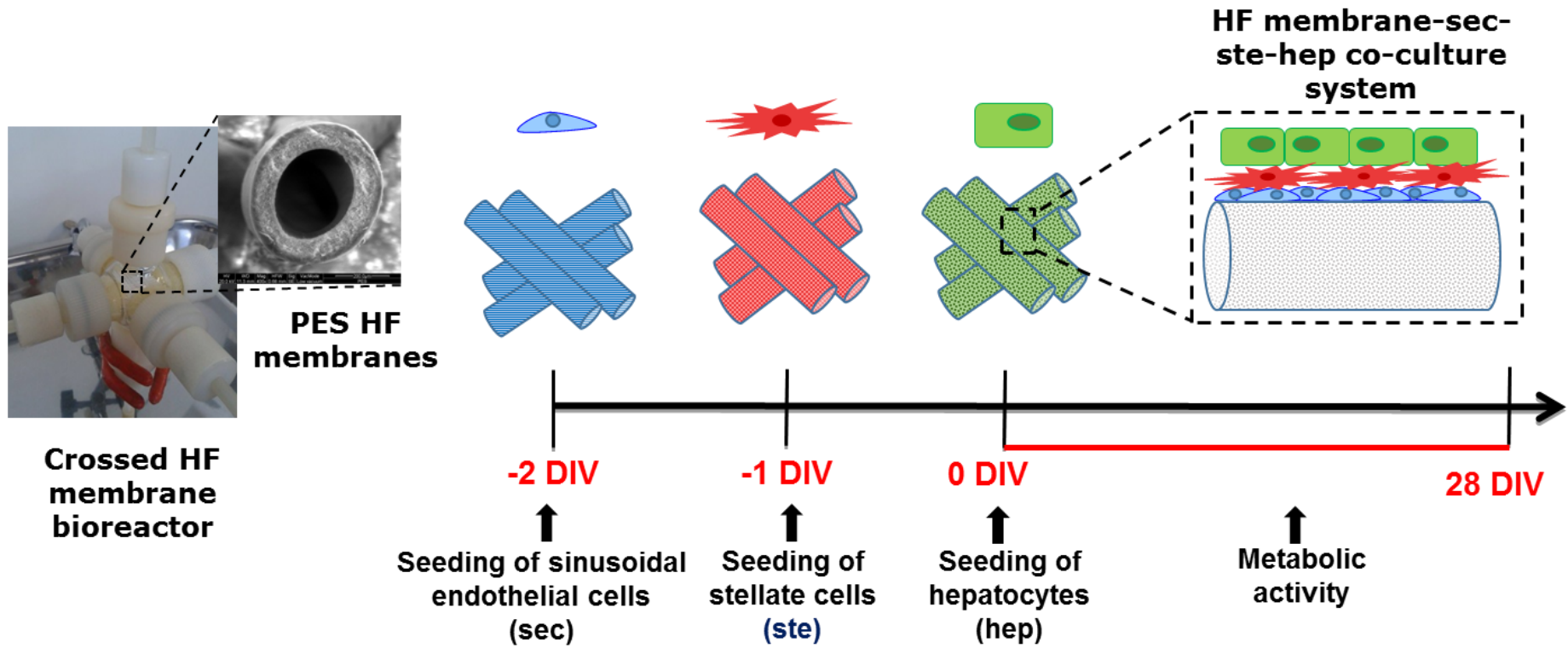


Fig. 3.1. Schematic representation of the organotypic HF membrane system. Bar scale in SEM image= 200 μ m.

Long-term co-culture experiments were performed under dynamic conditions in the HFMBR. Cells were incubated at 37° C in 5% CO₂ with 95% relative humidity for the duration of the experiments. Medium was changed every 3 days and sample were collected for analysis.

3.2.3 Cell Morphology

Cell morphology in the HF membrane system was evaluated by light-inverted microscopy, Scanning Electron Microscopy (SEM, Quanta 200F, FEI, USA) and Laser Confocal Scanning Microscopy (LCSM, Fluoview FV300, Olympus Italia) analyses. Samples were examined and representative images displaying the cell features in 3D structures were obtained at different culture time.

3.2.3.1 Sample preparation for SEM

Specimens of cell cultures were prepared for (SEM) by fixation in 2.5% glutaraldehyde, pH 7.4 phosphate buffer, for 30 minutes; followed by post-fixation in 1% osmium tetroxide and by progressive dehydration in ethanol.

3.2.3.2 Staining for LCSM

Cells were fixed using 4% paraformaldehyde for 20 minutes. Afterwards, the cells were permeabilized with 0.5% triton X-100 for further 20 minutes. Hepatocytes in the 3D HF membrane system were visualized for the albumin while stellate and sinusoidal endothelial cells were visualized for desmin and CD31, respectively. Albumin was detected by using specific goat monoclonal antibodies (Bethyl laboratories, Alabama, United States), diluted 1:200 in 2.5% normal donkey serum (NDS) and a CyTM5-conjugated AffiniPure donkey anti-goat IgG (Jackson ImmunoResearch Europe Ltd, Cambridge, UK) were used. CD31 was visualized by using a specific mouse monoclonal antibody (BD Biosciences, Franklin Lakes, NJ US) (diluted 1:200 in 2.5% NDS) raised against the glycoprotein CD31 of human origin and a CyTM3-conjugated AffiniPure donkey anti-mouse IgG (Jackson ImmunoResearch Europe Ltd, Cambridge, UK). Desmin was detected by using specific polyclonal rabbit antibodies (Thermo Fischer Scientific, Illinois, USA) diluted 1:25 in 2.5% NDS and a CyTM2-conjugated AffiniPure donkey anti-rabbit IgG (Jackson ImmunoResearch Europe Ltd, Cambridge, UK). All primary and secondary antibodies were incubated for 2 and 1.5 hours at room temperature, respectively. Counterstaining for nuclei was performed via incubation with DAPI 0.2 µg/ml (Molecular Probes Inc, Eugene, OR) for 30 min. Finally, samples were washed, mounted and viewed with LCSM (Fluoview FV300, Olympus Italia).

3.2.4 Assay of metabolic functions

3.2.4.1 Biochemical and ELISA assays

Samples of the culture medium were collected in pre-chilled tubes and stored at - 20° C until assayed. The urea concentration was assayed by the enzymatic urease method (Sentinel, Milan, Italy).

Albumin production in the samples was measured by the immunoenzymatic method (ELISA) using antibodies against human albumin. Ninety-six well plates were coated with 50 µg/ml monoclonal anti-albumin antibody (Bethyl laboratories, Alabama, US) (diluted 1:100) and left at room temperature for one hour. This was followed by blocking using 10% BSA solution overnight at 4°C. Afterwards, 100 µl of cell culture supernatant was added to the wells and incubated at room temperature for one hour. Following this step, horseradish peroxidase-conjugated secondary antibody against albumin (Bethyl laboratories, Alabama, US) (diluted 1:10000) was added for one hour at room temperature. The substrate buffer containing tetramethylbenzidine and H₂O₂ (Sigma, St. Louis, MO, USA) was added for 10 min. The reaction was stopped with 0.18 N H₂SO₄. Absorbance was measured at 450 nm using a Multiskan Ex (Thermo Lab Systems).

3.2.4.2. HPLC analysis of diazepam and metabolites

Diazepam elimination and its metabolite formation were assessed by incubating hepatocytes with 10 µg/ml diazepam. HPLC was used to assess the diazepam and metabolite concentrations in medium samples. The samples from the culture medium were alkalinized with 20% of 4M NaOH, precipitated with isopropanol (1:10) and extracted with ethyl acetate (5:1) by gentle rocking for 15 min and subsequent centrifugation at 200g for 15 min at RT. Thereafter, the ethyl acetate phase was evaporated and exsiccated under vacuum condition and the pellet was dissolved in mobile phase consisting of acetonitrile/methanol/0.04% triethylamine pH 7.04 at proportion of 25/35/40, respectively. Then, samples were HPLC-analysed using a C18-RP Purospher Star 5µm, 250x4.6 mm column, equipped with a precolumn (Merck KGaA, Darmstadt, Germany). The sample injection volume was 20µl. The mobile phase was delivered at 0.8 ml/min and the column was operated at ambient temperature. The effluents were monitored with a UV detector at 236 nm. Besides diazepam its metabolites temazepam and nordiazepam were detected. For all substances calibration curves were run regularly between 10 ng/ml and 10 µg/ml.

3.2.4.3. Dissolved oxygen concentration measurement

Dissolved oxygen concentration data was measured in HF membrane bioreactor by using a flow-through oxygen cells (Presens, Regensburg, Germany). The flow-through chemical optical oxygen sensors, based on the

quenching of luminescence by oxygen, were incorporated into the culturing perfusion loop at the inlet and outlet of the bioreactor. The sensors do not consume oxygen, are independent of the flow rate and maintain long-term stability. A fiber optic cable transmitted the optical signal between the sensor and the Fibox 4 portable oxygen meter (PreSens GmbH, Germany); The transmitter was later connected to a PC and via the software the data collected during the experiment was exported to excel sheets. The oxygen probes detected oxygen concentration with resolution of $\pm 0.4\%$ O₂ at 20.9% O₂ and a response time <30s. The membrane bioreactor containing cells was cultured in a CB 150 CO₂/O₂ incubator (BINDER, Germany).

3.2.5. Statistical analysis

The statistical significance of the experimental results was calculated using Student's t-test and ANOVA test followed by a Bonferroni t-test ($p < 0.05$).

3.3. Results

3.3.1 Morphological evaluation of organotypic membrane system

A sequentially-layered organotypic liver system was created on modified PES hollow fiber membranes (Fig. 3.1). These membranes have an internal diameter and wall thickness of $300 \pm 40 \mu\text{m}$ and $100 \pm 25 \mu\text{m}$, respectively and mean pore size of $0.2 \mu\text{m}$. They have a hydrophilic character ($\text{WCA} = 43 \pm 3.5^\circ$) and hydraulic permeance of $15.2 \text{ L/m}^2\text{h mbar}$. The hollow fiber membranes provided a wide surface area for the adhesion of cells that assembled in a 3D structure. Morphological analysis of the organotypic culture through light microscopy showed the formation of tissue-like structures around and between fibers at day 13 of culture (Fig 3.2).

The progression of this organization process is shown by SEM micrographs. Fibers appeared completely covered by cells that formed small aggregates reaching 1 mm in size in some points as a result of cell self-assembling and the growth process undertaken by cells starting from the initial cell suspension (Fig 3.3). Immunohistochemical staining of markers for different liver cell types was performed to evaluate the cell arrangement and composition in the 3D liver cell system. Hepatocytes and non-parenchymal cells formed tissue-like aggregates between the capillary fibers. CD31 staining for endothelial cells showed the formation of tube-like structures surrounding the hepatocytes, which were recognized by albumin staining, as shown in Fig 3.4a. Actin was found in a filament pattern that formed densely packed bundles along the edges of the

plasma membrane. Hepatocytes appeared in a liver cord like organization. Staining of desmin was used to detect stellate cells, which showed a myofibrous structure typical of activated stellate cells (Fig. 3.4b). The expression of the specific markers confirmed the presence of the different cell types that reorganize themselves in the 3D liver system producing a complex cytoarchitecture vascularized by the tube-like structure formed by sinusoidal endothelial cells, which are visible in Fig. 3.4c.

3.3.2 Hepatocyte-specific functions

In order to verify the positive impact of non-parenchymal cells on the maintenance of hepatocyte functions, homotypic and organotypic cultures on the hollow fiber membranes were first investigated in batch system evaluating hepatocyte specific functions in terms of albumin production and urea. Functional analysis of liver specific metabolic activity showed that hepatocytes maintained their functions investigated throughout 13 days of batch co-culture (Fig. 3.5). Urea synthesis rate of the organotypic cultures was higher than that of the homotypic cultures throughout the experiment as shown in Fig 3.5a.

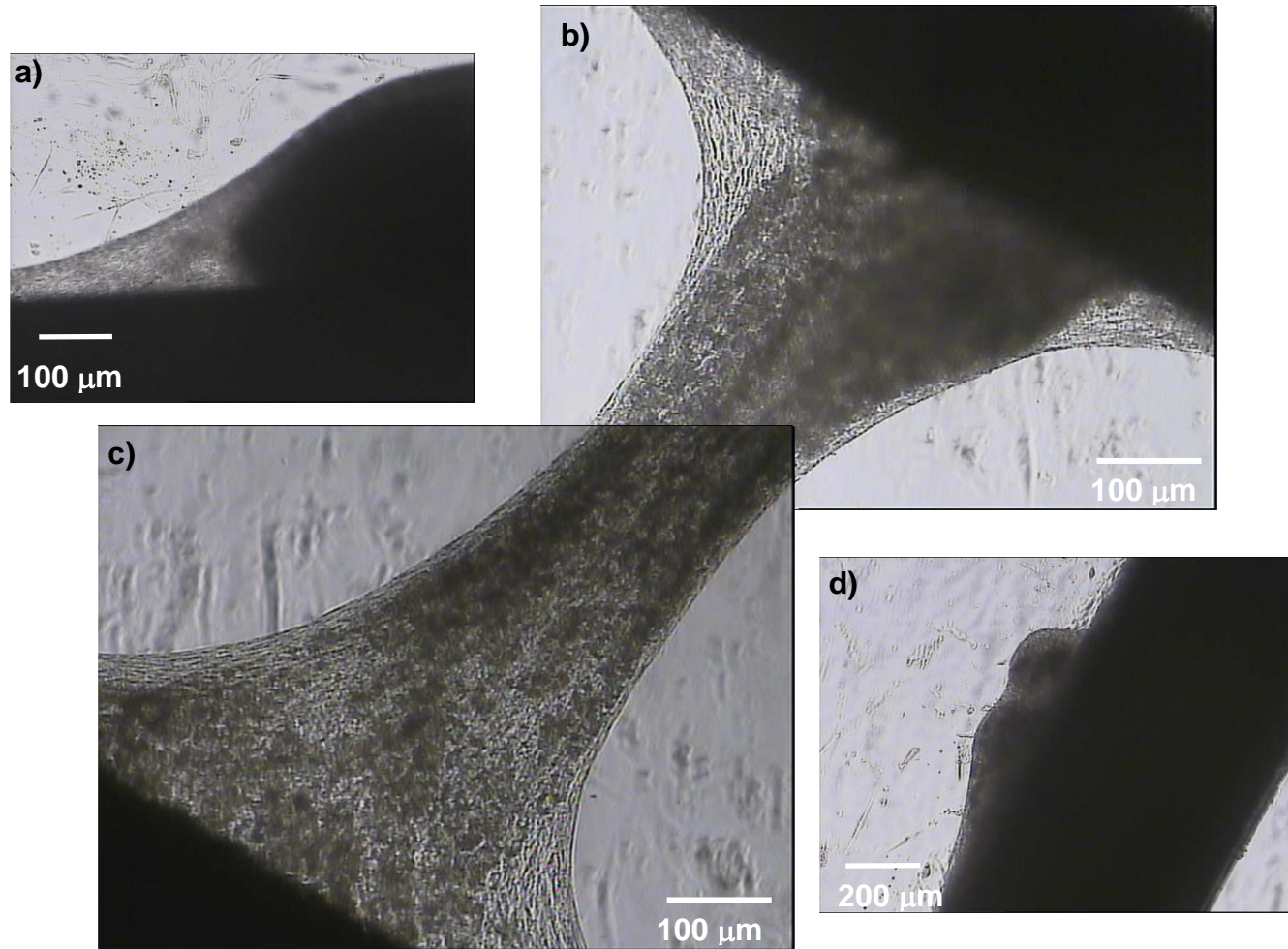


Fig. 3.2. Light Microscope images of the 3D structure formed by cells around (a,d) and between (b,c) the fibers in organotypic hollow fiber membrane system at day 13 of culture.

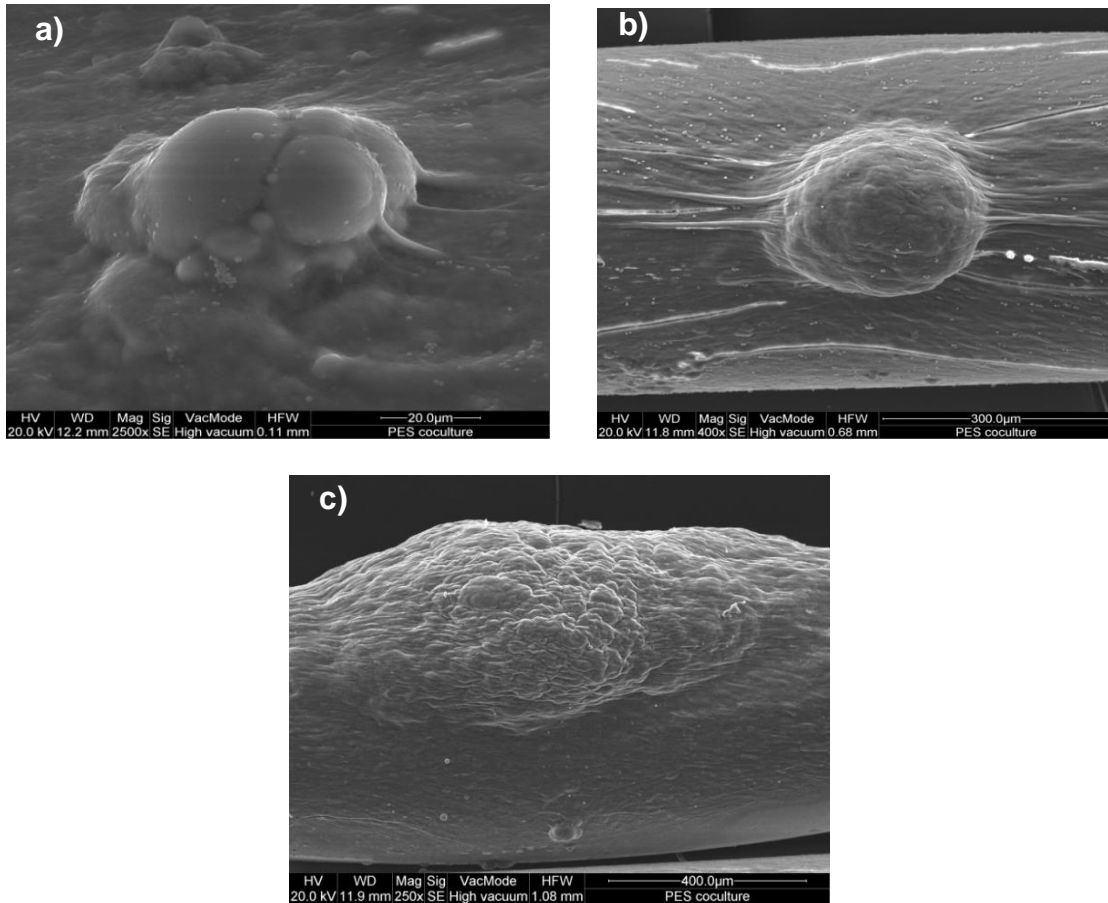
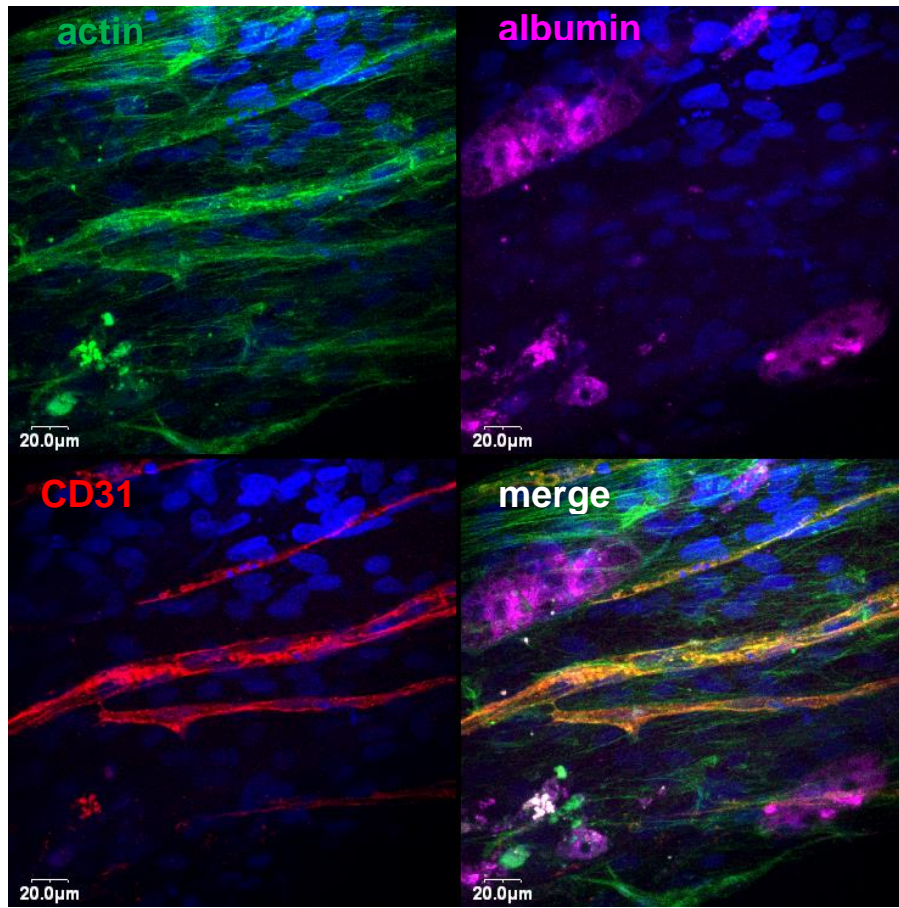


Fig. 3.3. Scanning Electron Microscope (SEM) micrographs of cells in organotypic culture on PES hollow fiber membranes at different magnification. (a-c) Aggregates formed by stellate, sinusoidal cells and hepatocytes around fibers at day 17.

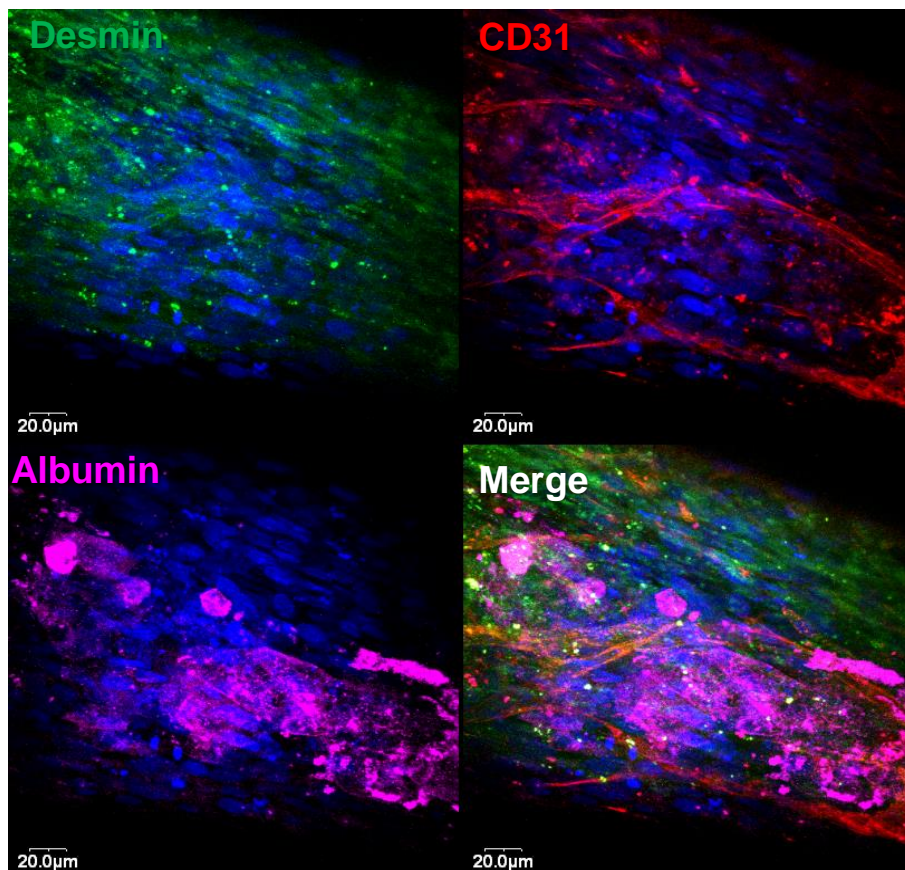
The initial high rate of urea synthesis detected at day 2 in both systems is probably due to the use of serum-containing medium on the first day of culture, which may contain ammonia and amino acids that can be eventually converted by hepatocytes into urea. Afterwards the cells were cultured in serum-free medium. The organotypic cultures maintained an almost stable urea synthesis rate from day 8 until the end of the experiment around values of $4.6 \pm 0.1 \mu\text{g/h}/10^6$ cells; whereas the homotypic cultures showed a progressive decline of urea synthesis rate to values of $0.5 \pm 0.3 \mu\text{g/h}/10^6$ cells at day 13.

Albumin synthesis rate, on the other hand, increased with time in both organotypic and homotypic systems as shown in Fig 3.5b. However, hepatocytes in the organotypic system synthesized albumin with rates significantly higher than those of the homotypic one throughout the experiment. The average albumin synthesis rate in case of organotypic culture from day 8 onwards was $260 \pm 25 \text{ ng/h}/10^6$ cells compared to an average of $116 \pm 17 \text{ ng/h}/10^6$ cells for the homotypic culture over the same period.

A)



B)



C)

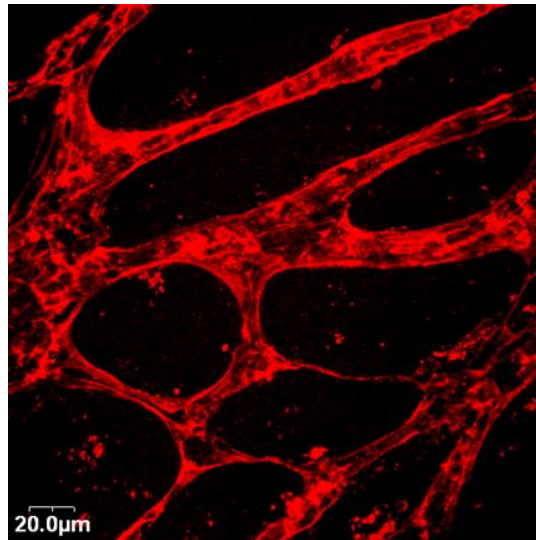
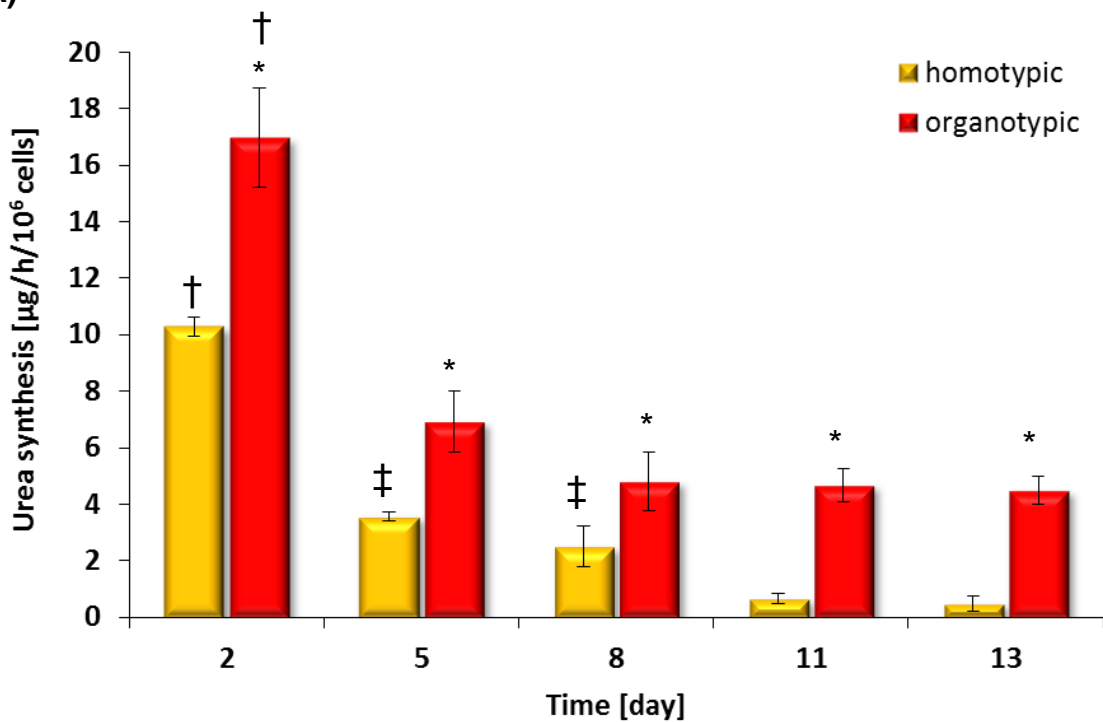


Fig. 3.4. LSCM images of human hepatocytes, sinusoidal and stellate cells in organotypic cultures on PES hollow fiber membranes at day 13. A) Cells were stained for the actin (green), albumin specific for hepatocytes (magenta) and for the cell adhesion receptor CD31 specific for sinusoidal endothelial cells (red) and nuclei (blue). B) Cells were stained for intermediate filament protein desmin (green) specific for stellate cells, for albumin (magenta), the cell adhesion receptor CD31 (red) and nuclei (blue). C) Tube-like structures formed by sinusoidal endothelial cells in the organotypic membrane system. Cells were stained for the cell adhesion receptor CD31 (red).

A)



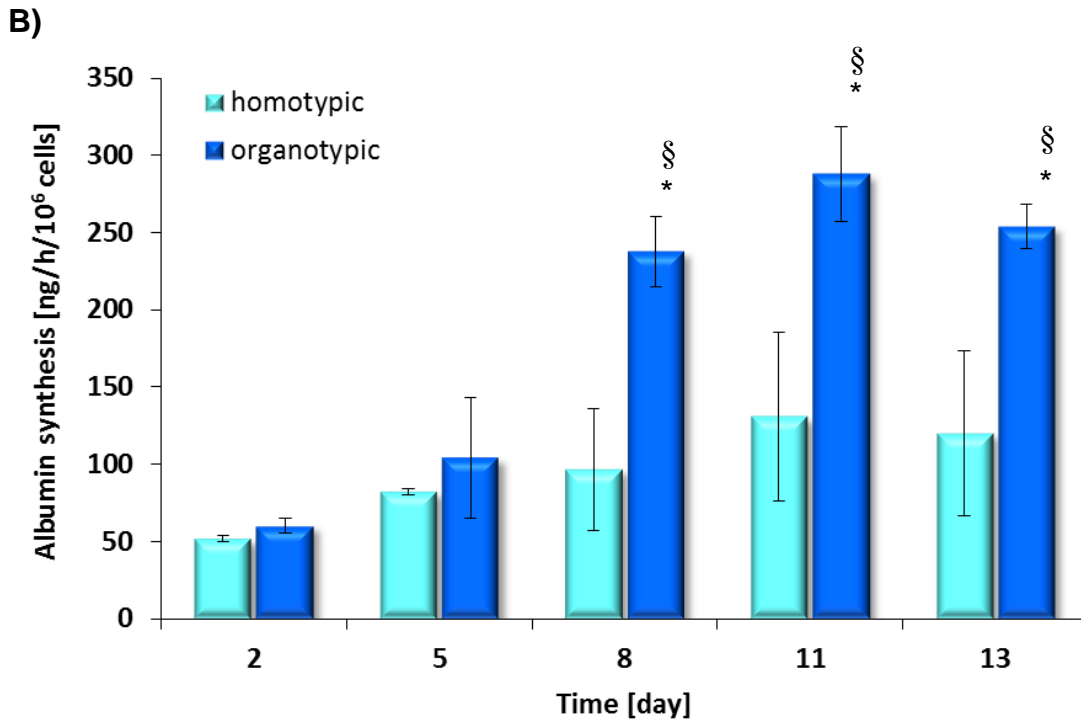


Fig. 3.5. Liver specific functions of primary human hepatocytes in homotypic and organotypic batch HF membrane systems: (a) urea and (b) albumin synthesis. The values are the mean of three experiments \pm standard deviation. Data statistically significant $p < 0.05$: (*) vs homotypic at the same day of culture according to student's t-test; (†) vs day 5, 8, 11 and 13 for the same membrane system; (‡) vs day 11 and 13 for the same membrane system; (§) vs day 2 and 5 for the same membrane system according ANOVA followed by Bonferroni t-test.

A different trend was observed for diazepam metabolism: hepatocytes in both systems exhibited similar biotransformation activity for the first 8 days, after which a strong increase of diazepam metabolism was measured with rate values significantly higher than those of homotypic ones (Fig 3.6a). Nordiazepam (NDZ) and Temazepam (TMZ) phase I metabolites were detected; with TMZ being the major metabolite. DZP biotransformation enhanced over time for both cultures. In the case of homotypic culture, the TMZ and NDZ formation rate reached maximum values of 191 ± 98 and 36 ± 16 ng/h/ 10^6 cells at day 13, respectively (Fig. 3.6b). TMZ and NDZ were produced in the organotypic membrane system with rates of 312 ± 39 and 74 ± 25 ng/h/ 10^6 cells, respectively at day 11 (Fig. 3.6a). This system maintained the ability to metabolize diazepam through the production of TMZ and NDZ with rates of 283 ± 36 and 65 ± 12 ng/h/ 10^6 cells, respectively at day 13.

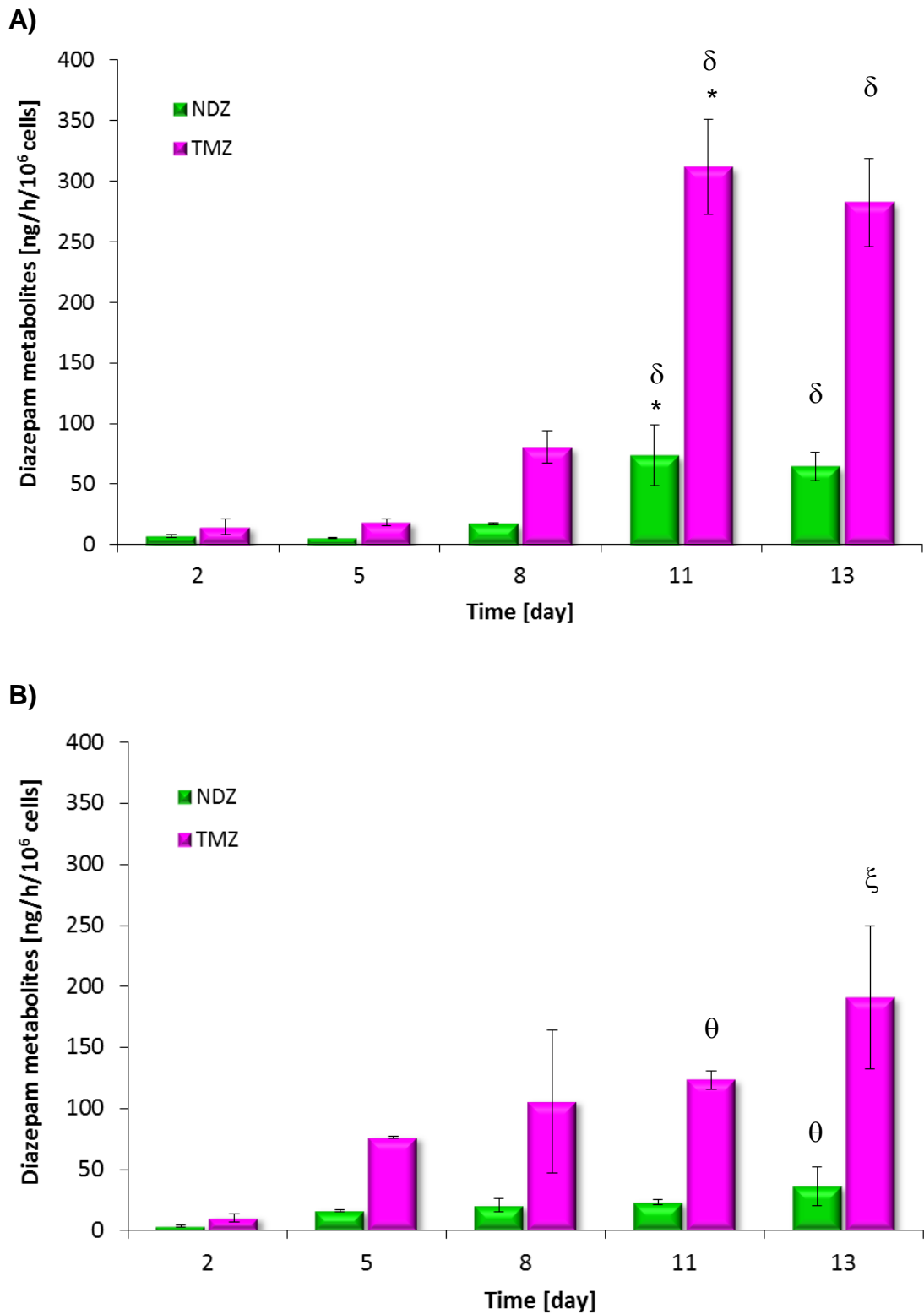
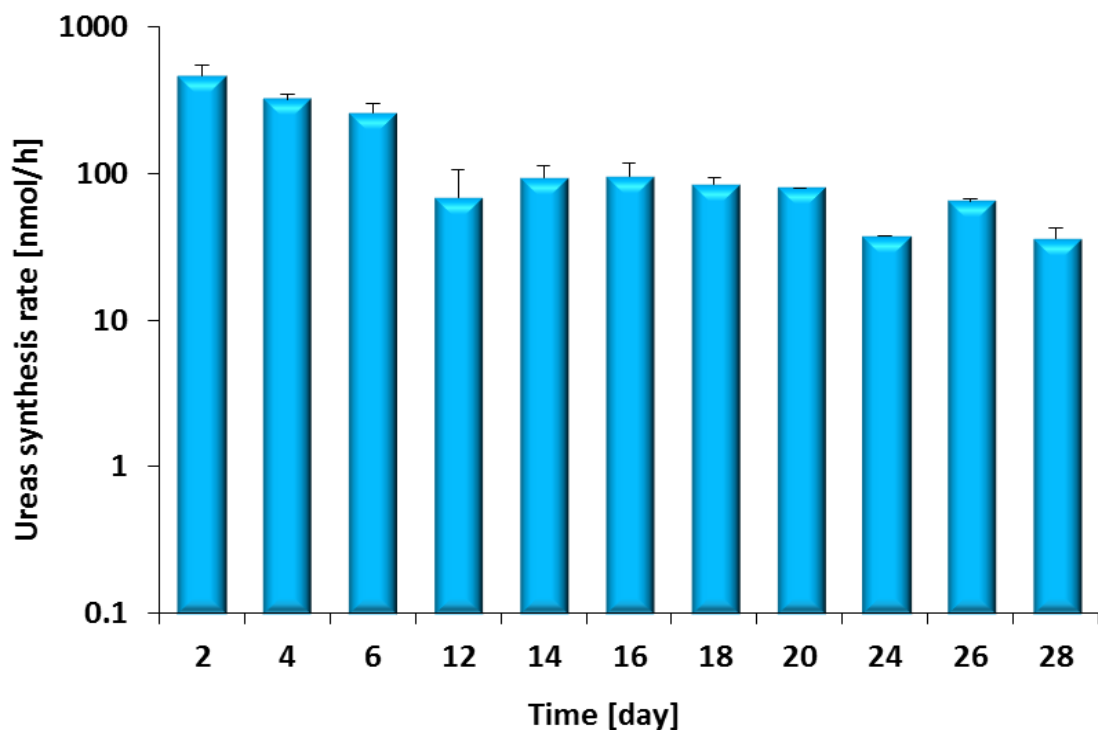


Fig. 3.6. Diazepam metabolite formation: nordiazepam (NDZ) and temazepam (TMZ) by primary human hepatocytes in (a) organotypic hollow fiber membrane system and in (b) homotypic HF membrane system. The values are the mean of three experiments \pm standard deviation. Data statistically significant $p < 0.05$: (*) vs homotypic at the same day of culture according to student's t-test; (θ) vs day 2, (ξ) vs day 2 and 5, (δ) vs day 2, 5 and 8 for the same membrane system according ANOVA followed by Bonferroni t-test.

After the evaluation of batch cultures, the organotypic system was created in the HF membrane bioreactor. In this device cells were co-cultured for 28 days and quantitatively assessed the stability of their liver specific functions. Albumin secretion increased during the first days of culture with time and then remained at values of 5-6 pmol/h up to almost 1 month (Fig. 3.7a). Consistently also urea synthesis was maintained for all culture time at a level of 80-90 nmol/h although the highest metabolic rates were measured in the first days owing to the presence of serum in the culture medium (Fig. 3.7b). To assess the biotransformation functions of human organotypic system in the bioreactor diazepam was administered in the time and evaluated the production of metabolites formed by the phase I reactions catalyzed by P450 cytochrome. Nordiazepam (NDZ), temazepam (TMZ) and oxazepam (OXZ) metabolites were detected; with TMZ being the major metabolite. Diazepam biotransformation enhanced over time reaching highest values of 1209 ± 138 and 169 ± 11.2 pmol/h at day 24 for TMZ and OXZ, respectively, as highlighted by the time profiles of the diazepam metabolite formation in the bioreactor (Fig. 3.8).

A)



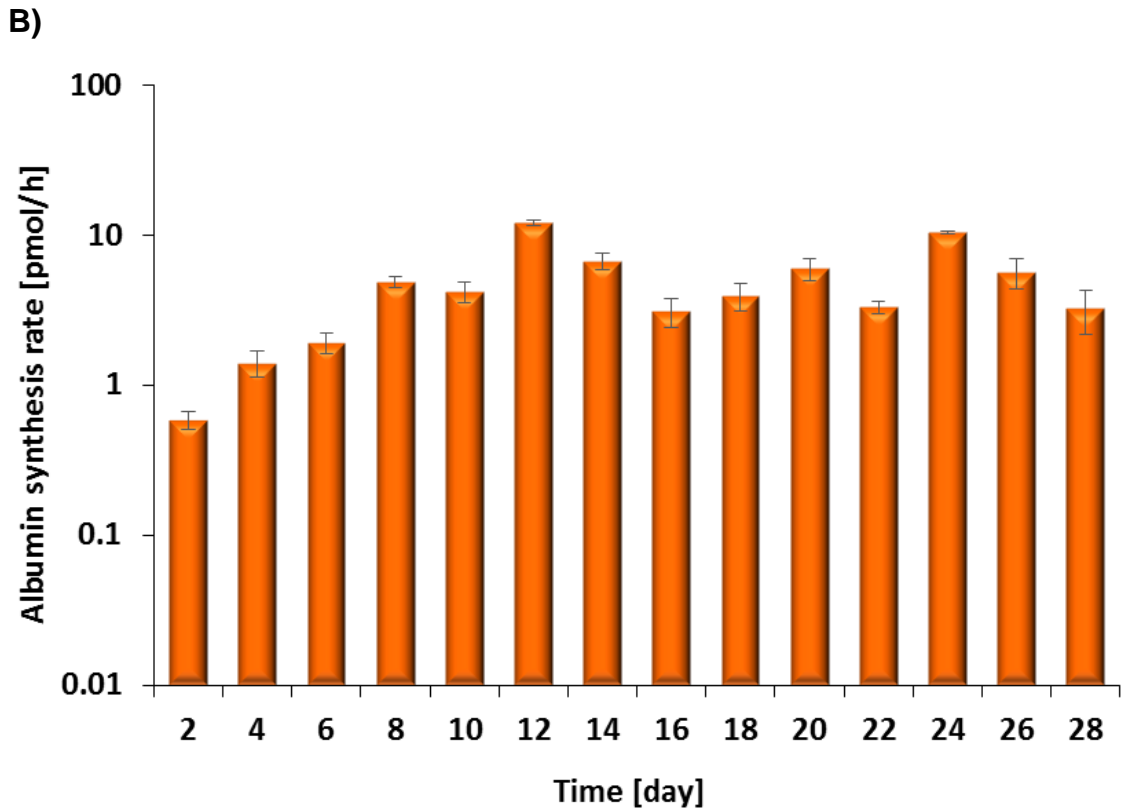


Fig.3.7. Liver specific functions of primary human hepatocytes in organotypic HF membrane bioreactor: (a) urea and (b) albumin synthesis. The values are the mean of three experiments \pm standard deviation.

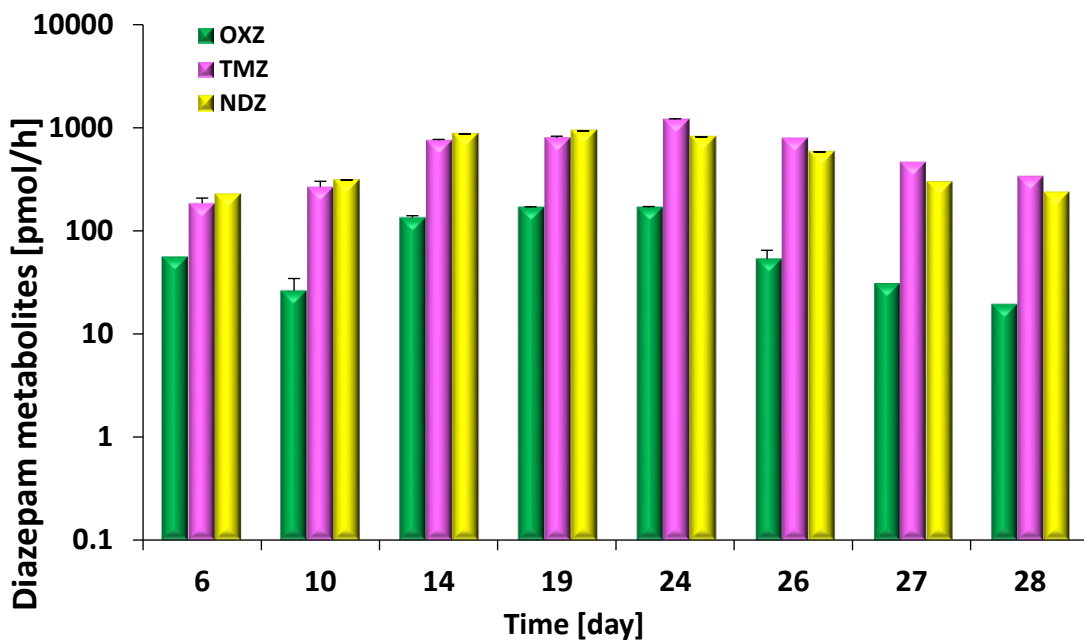


Fig. 3.8. Diazepam metabolite formation: nordiazepam (NDZ) and temazepam (TMZ) and oxazepam (OXZ) by primary human hepatocytes in organotypic HF membrane bioreactor. The values are the mean of three experiments \pm standard deviation.

3.3.3 Oxygen uptake rate

Considering the importance of oxygen supply for cells and that hepatocytes are particularly sensitive to the availability of oxygen, the concentration of this nutrient dissolved in the culture medium was monitored throughout the investigated time in both batch and bioreactor experiments. In batch experiments, Hepatocytes displayed a relatively stable oxygen uptake rate (OUR) and at levels that implied the maintenance of the oxygen concentration at around 20% in the atmosphere. Indeed, the oxygen uptake rate in the homotypic system was highest on the first 2 days, averaging around 664.8 ± 15 nmol/h, then it became almost stable for the rest of the experiment at values of 180 ± 70 nmol/h, as shown in Fig. 3.9. Oxygen concentration in the case of organotypic cultures decreased throughout the experiment as seen in Fig 3.10 leading to reaching a critical oxygen concentration of $50 \mu\text{mol/L}$ which is considered the threshold for cell survival. To circumvent this problem, the oxygen concentration in the atmosphere of culture chamber was increased sequentially to account for the increasing oxygen consumption by the cells. OUR clearly shows the increasing oxygen consumption by the cells reaching a maximum of approximately 3000 nmol/L at day 11 and the subsequent drop in oxygen consumption that coincides with the increase of oxygen concentration in the incubator (Fig. 3.9).

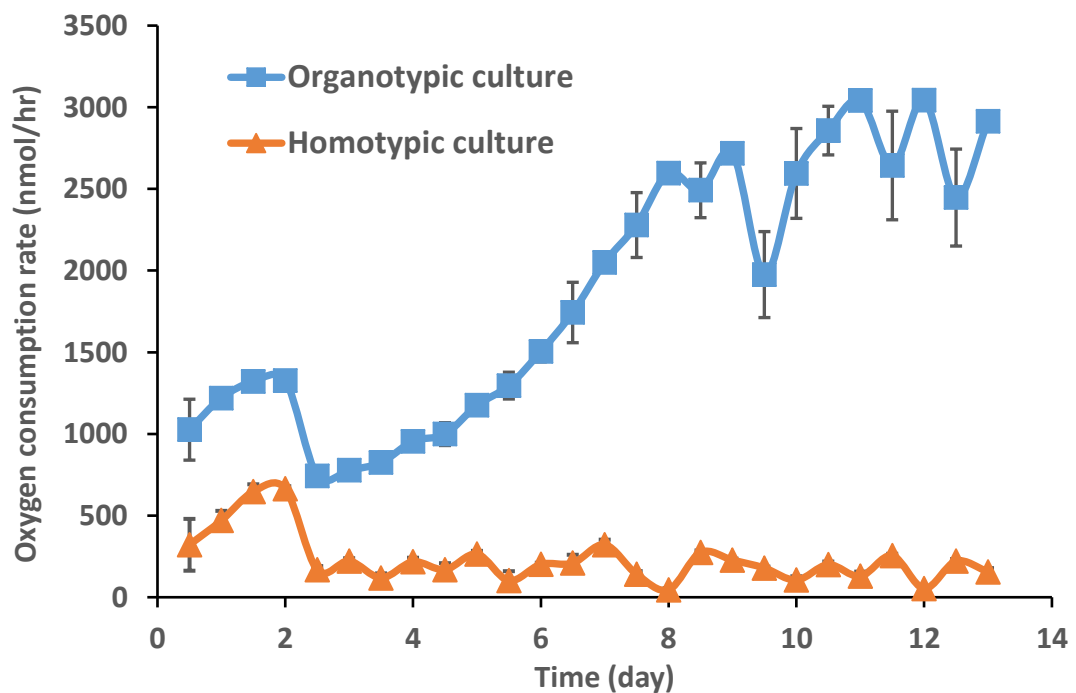


Fig. 3.9 Oxygen consumption rates in organotypic (blue) and homotypic (orange) HF membrane systems. The values are the mean of three experiments \pm standard deviation.

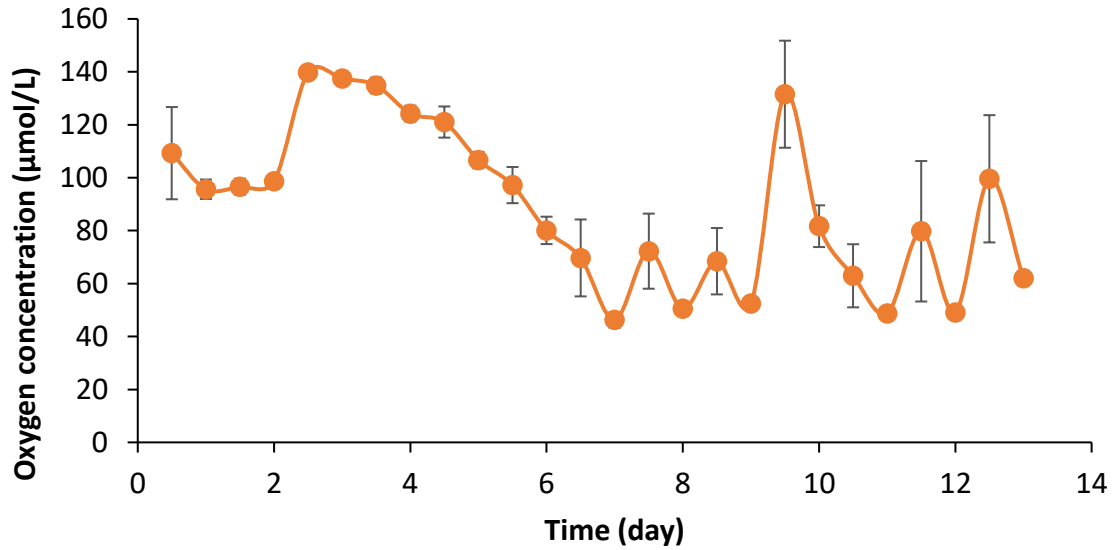


Fig. 3.10. Oxygen concentration of human organotypic cultures, cultured on PES HF.

On the other hand, cells in bioreactors exhibited a steadier oxygen supply as shown in Fig. 3.11, in which the oxygen concentration measured in the outlet ranged between 150 and 190 nmol/L. Oxygen consumption rates fluctuated between approximately 900 and 1900 nmol/L throughout the experiment (fig.3.12). Cells displayed rates of oxygen consumptions at levels that implied the maintenance of the oxygen concentration at around 20% in the atmosphere without any oxygen limitation.

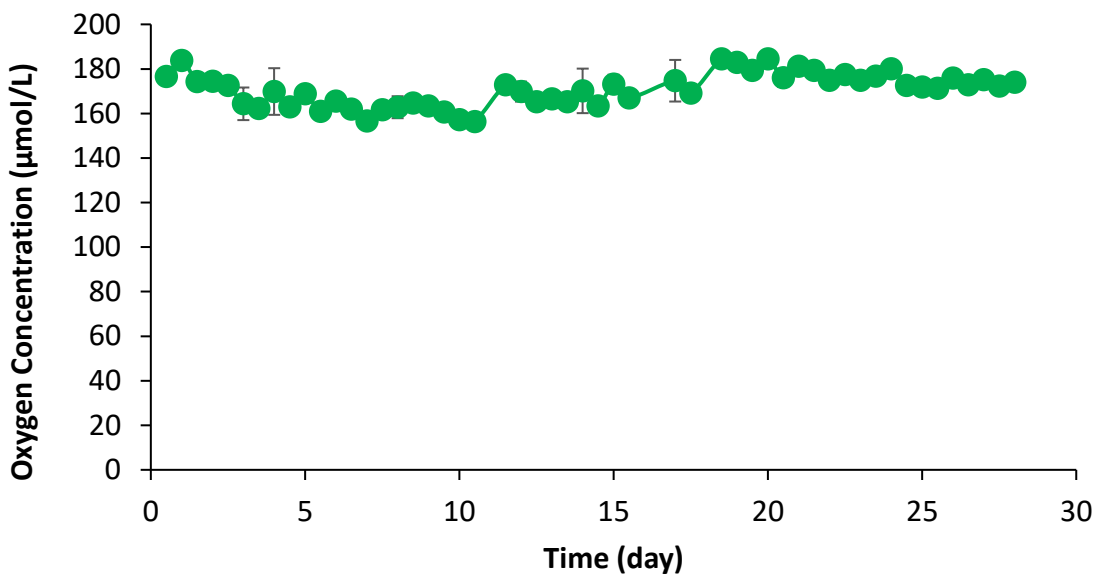


Fig. 3.11. Oxygen concentration of human organotypic liver cultures, cultured in a PES/PES crossed HFMBR, measured using a flow-through oxygen sensor placed at the outlet of the bioreactor.

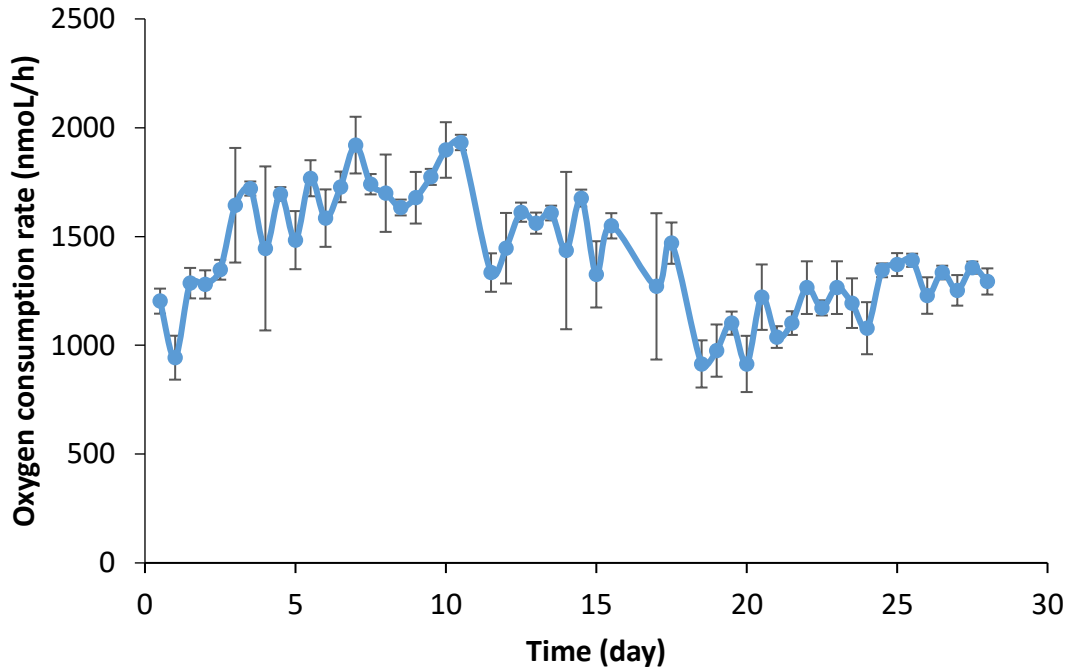


Fig. 3.12. Oxygen consumption rates of human organotypic liver cultures, cultured in a PES/PES crossed HFMBR.

3.4. Discussion

The development of an in vitro liver tissue with a complete ultra-architecture remains a critical issue in tissue engineering. Several techniques and methodologies have been extensively applied to liver tissue engineering to reach this goal. Developing more in vivo-mimicking structures will ultimately require chemical and mechanical cues including heterocellular interactions of parenchymal and non-parenchymal cells, which play a pivotal role in tissue morphogenesis and development. Recent studies have shown the beneficial effect of heterotypic interactions on liver specific functions highlighting the role of non-parenchymal cells in the maintenance of hepatocytes phenotype and functions (59, 225, 226)

To engineer liver ultrastructure, it is important to consider the use of hepatocytes together with non-parenchymal cells to create a structurally and functionally active heterocellular construct. In this study, we explored a human organotypic hollow fiber membrane system in batch and in dynamic conditions by using primary human stellate cells, sinusoidal endothelial cells and hepatocytes, which were sequentially seeded in order to mimic the liver layers. In this respect, the use of human liver cells is very important because only these cells can respond as would the human liver; cell lines and animal cells are ultimately poor substitutes, exhibiting features that are often quite distinct from those of the human cells. Our strategy was to develop a human liver membrane system and

to evaluate the morphological, phenotypic and functional characteristics of cells in comparison with homotypic cultures consisting only of hepatocytes. In this context, hollow fiber membranes are particularly advantageous because they provide a wide area for cell adhesion and a 3D structure on which to build a tissue. Additionally, in the bioreactor they allowed the compartmentalization of cells in a well-controlled microenvironment at molecular level thanks to their selective permeability properties that govern mass transfer exchange.

The modified PES membrane surface acted as a support allowing cell adhesion and subsequent formation of tissue-like structures ranging in size from 250 μm to over 1 mm as the morphological analysis highlights. The immunohistochemical studies confirmed the tissue-like arrangement of parenchymal and non-parenchymal liver cell populations in the organotypic culture. Hepatocyte aggregates contained tube-like structures that stained positive for the endothelial cell marker CD31, and stellate cells as detected by the staining for desmin. Since the inoculated cell suspension predominantly contained single cells and small cell clusters, the finding of large tissue-like cell associations within the membrane system indicates active reorganization of the cells in the culture system rather than pure transfer of those structures from the cell suspension. The formation of aggregates around the fibers is due to the contribution of stellate cells that facilitate the aggregation (227). It is known that stellate cells both physically and chemically interact with hepatocytes *in vivo*. They lie in the perisinusoidal space in the liver and also produce hepatocyte growth factor (HGF), transforming growth factor- α (TGF- α) and epidermal growth factor (EGF), three potent growth factors for hepatocyte proliferation during liver regeneration (36). The potent mitogens, Insulin-like growth factor and platelet-derived growth factor, are also secreted by stellate cells (228). Collectively, these factors allow hepatic stellate cells to influence their own gene expression and phenotype as well as that of other cells of the liver.

In return, hepatocytes influence stellate cells by expressing insulin-like growth factor I that activates stellate cell proliferation *in vitro*. In the organotypic system, the formation of aggregates could be due to the physical contact between the cell types but also to chemotaxis mechanism through the paracrine factors. In the 3D multicellular construct sinusoidal endothelial cells developed around the fibers tube-like structures, which resembled those found in human liver tissue. These findings clearly indicate that a physiological cell reorganization process occurred *in vitro*.

The co-culture of sinusoidal endothelial cells and hepatic stellate cells together with hepatocytes preserved 3D structural architecture of the construct and improved the liver-specific functions. Both albumin and urea synthesis rates were significantly higher in the organotypic HF membrane system with respect to the hepatocyte monoculture. These results are in line with the previous published data, regarding human hepatocytes co-cultured with only endothelial cells (59,

229) and are also consistent with many other published co-culture findings regarding human and rat hepatocytes in co-culture with non-parenchymal cells (225, 226). In this study, the designed approach of sequentially seeding the three different human cell types on HF membranes, for the development of a 3D liver layered system, improved the expression of metabolic functions due to the crosstalk between hepatocytes, sinusoidal and stellate cells. The rates of albumin and urea synthesis measured in the organotypic hollow fiber membrane system were 2-fold and 6-fold (day 13), respectively, of the membrane system containing only hepatocytes. Although the mechanisms by which co-culture of hepatocytes with other cell types induce and stabilize liver-specific functions are not completely defined; in the case of albumin, it was found to be regulated transcriptionally and the level of transcription of the corresponding mRNA was stabilized at higher rates in rat hepatocyte co-culture (230). Considering that liver specific functions can be modulated through several potential mediators of cell communication in co-cultures (receptors, gap junctions, cytokines and ECM), many distinct mechanisms may operate in concert, each modulating a subset of hepatospecific functions. Additionally, the establishment of an organotypic culture in the crossed HF membrane is correlated with the maintenance of the metabolic activity of cells over an extended period of time. The membrane bioreactor created a physiologically relevant microenvironment for cells since it ensures a continuous perfusion of nutrients and metabolites that permeate through the porous wall of feeding fibers from the lumen to the extracapillary space where cells are cultured. In the meantime, catabolites and specific cellular products are removed from the extracapillary space through the removing fibers. In addition, the crossed configuration allows a more efficient packing of hollow fibers (231). Our data highlights that human liver cells have well preserved the biotransformation activity in the 3D HF membrane bioreactor. The diazepam metabolic activity achieved in the organotypic membrane bioreactor is a further confirmation of the establishment of a more closely in vivo microenvironment that results in an increase of CYP activity with time. In particular, the temazepam formation is catalyzed by CYP2C19, 3A4 and 3A5 through 3-hydroxylation reaction, and the nordiazepam is catalyzed by CYP2B6, 2C8, 2C9, 2C18, 2C19, 3A4 and 3A5 through N-demethylation reaction (232, 233). Both metabolites are then N-demethylated and 3-hydroxylated respectively to the oxazepam. The maintenance of diazepam biotransformation rates over an extended period of almost 1 month has proved the stability of the specific CYP isoenzymes involved in their formation in the dynamic organotypic membrane system.

In the tissue engineering constructs, oxygen is usually the limiting nutrient owing to its weaker solubility in aqueous medium compared with, for example, glucose. The oxygen uptake rate data of cells in the organotypic membrane bioreactor underlines the viability of cells inside the construct. Oxygen uptake rate (OUR) was constant in the time for hepatocyte monoculture system while increased in

the case of organotypic system because of the presence of sinusoidal and stellate cells, which are proliferating cells. The increase of OUR in the organotypic membrane system involved a decrease of oxygen concentration into the medium even though it was maintained well above the critical threshold value of 5%, which is considered the minimum for cell survival (234). Since the average physiological oxygen tensions in the liver lobule range from 9-11% oxygen in the periportal zone to 4–6% oxygen in the hepatovenous zone, the actual oxygen concentration at the cellular level in the 3D organotypic system was maintained at levels compatible with their metabolic functions.

Oxygen uptake rate (OUR) increased with time because of the presence of sinusoidal and stellate cells, which show proliferation activity during the first days of culture. The actual oxygen concentration at the cellular level in the 3D organotypic bioreactor was maintained at levels compatible with their metabolic functions. A rational design of 3D liver model oriented to in vitro and in vivo applications must ensure an efficient mass transfer of nutrients, especially of oxygen. The organotypic HF membrane bioreactor system containing the oxygen sensors allowed accurate and extensive measurements of the cell oxygen uptake rate throughout the culture time. This information is relevant for the optimization of a 3D liver model and for the biofabrication of tissues and/or organs in order to avoid oxygen limitations and to maximize metabolic functions.

3.5. Conclusions

In conclusion, a 3D human liver system was developed by using a designed approach based on the sequential seeding of primary human sinusoidal endothelial cells, stellate cells and hepatocytes on modified PES HF membranes in static and dynamic conditions. Cells reorganize themselves in the 3D liver HF membrane system producing a complex cytoarchitecture with the presence of tube-like structure formed by sinusoidal endothelial cells. The organotypic system was maintained functionally active in the HF membrane bioreactor, which allowed a continuous perfusion of cells and the selective mass transfer of molecules to and from cell compartment creating a physiologically relevant microenvironment. Urea and albumin synthesis as well as the diazepam biotransformation functions were performed at high levels up to 28 days. The oxygen uptake rate data underlines the viability of cells inside the organotypic membrane construct for an extended period of time and the efficiency of the bioreactor to provide an adequate oxygen supply to the cells.

CHAPTER 4

Expansion and differentiation of stem cells in the hollow fiber membrane bioreactor

4.1 Introduction

Extracorporeal liver support systems artificial liver (BAL) bioreactors have been proposed to replace some of the hepatic functions of the failed liver and support patients to recovery or bridge them to orthotopic liver transplantation (235). Initial clinical applications using either xenogeneic or allogeneic adult hepatocytes had been reported (158, 236, 237). Although the concept is accepted and safety, feasibility, and improvement of some clinical parameters have been demonstrated, the technology is not a standard therapy yet in clinics (238).

One remaining key issue is identifying the right cell source to charge BALs with. There are several candidate cell types: adult human hepatocytes, xenogeneic hepatocytes (mainly porcine) and human cell lines. Although porcine hepatocytes are more readily available, the concerns for potential zoonotic transmission, species differences, and immunological problems reduce enthusiasm for their clinical application (239). Immortalized human hepatocyte cell lines can provide an unlimited resource for BAL systems, (240) although concerns exist about their metabolic function and the transmission of immortalized cells or tumorigenic products to the patient's circulation (241).

Human somatic stem cells are also considered as an alternative cells source, as their intrinsic proliferative capacity and differentiation potential are attractive for BAL applications. There is no risk of zoonotic transmission or immunological problems such as those associated with xenogeneic hepatocytes. Since cells are not genetically modified, there is reduced risk of tumorigenicity by contrast to hepatoma cell lines or immortalized human cell lines. On the other hand, functional maturity represents issues of concern for mesenchymal stem cells (242).

Somatic stem cells for use in hepatogenic differentiation are categorized into extrahepatic and intrahepatic stem cells with respect to their origin. It has been reported that hematopoietic stem cells (243), umbilical cord blood mesenchymal stem cells (244), adipose-derived mesenchymal stem cells (245) and bone marrow mesenchymal stem cells (246) as extrahepatic stem cells can differentiate in vitro into hepatocyte-like cells. On the other hand, there are intrahepatic stem cells, which are thought to have a higher hepatogenic differentiation potential than extrahepatic stem cells due to their pre-existence in the liver microenvironment. These intrahepatic stem cells include oval cells, clonogenic epithelial cells, and mesenchymal stem-like cells.

Beside oval or clonogenic epithelial cells, recently hepatic stem cells with mesenchymal characteristics have been isolated (hHMSC). They differ from other resident hepatic stem/progenitor cells in appearance as well as marker expression. hHMSCs are spindle-shaped, whereas liver progenitor cells are oval. They express mesenchymal stem cell markers CD44, CD90 and CD105, and lack markers for hematopoietic stem and oval cells (142). Like other stem cells, hHMSC proliferate in vitro in the presence of serum allowing for expansion of these cells prior to their differentiation.

Traditionally, conventional cell culturing is performed on two-dimensional surfaces and with discontinuous medium exchange under static conditions. The static culture conditions are characterized by an unstirred medium layer overlying cells attached to a gas impermeable substratum and are exposed to changes of nutrient concentration and catabolite accumulation on time. This type of culture is often used to address basic scientific questions. However, it appears inadequate for research in regenerative medicine and clinical translation. Two major components are lacking in conventional culture that are required to provide a tissue-like environment: perfusion and three-dimensionality (247).

Therefore, a multicompartiment crossed hollow fiber membrane bioreactor was used, which provides dynamic medium and gas supply within a three-dimensional (3D) perfusion environment.

Our goal was to investigate for the first time the expansion and differentiation of hHMSCs in a 3D perfusion bioreactor, which was developed previously for the long-term maintenance of human hepatocytes. To this end, 22-week-gestational-age hHMSCs were used. The hepatogenic differentiation of cells was first explored by using different culture conditions in batch system with Collagen-I and fibronectin coating in comparison with the uncoated plates. Moreover, two differentiation protocols were examined. The first was described by Lee et. al. in 2004 (246), it described a 2-step differentiation protocol for hepatogenic differentiation of bone marrow-derived mesenchymal stem cells.

The second protocol was recently published for the hepatogenic differentiation of hHMSCs using a shorter 2 step-protocol that reportedly lead to liver-specific functions being observed after two weeks, as compared to 4 weeks in the first protocol (248). The optimized conditions were then used in the 3D perfusion bioreactor in order to establish uniform culture conditions that ensure continuous feeding of nutrients and growth factors and removal of catabolites. The expansion and differentiation depends on controlling key process variables: nutrient and metabolite concentrations, growth factor compositions, and physiological parameters (e.g., temperature, pH, and oxygen). The crossed HF membrane bioreactor creates a homogeneous environment for cell culture in which the concentrations of nutrients and metabolites are monitored and controlled and differentiation signals are provided to the cells. Selective exchange of gases and metabolites through the selective HF membranes

ensured a microenvironment adequate for the induction and maintenance of the liver-specific functions exhibited by hepatocytes.

4.2 Materials and Methods

4.2.1 Hollow-fiber membrane bioreactor

For the culture and differentiation of hHMSCs, the crossed HFMBR described earlier in chapters 2 and 3 was used.

The oxygenated medium enters from the reservoir to the membrane bioreactor with a flow rate Q_f of 0.6 ml/min that was set on the basis of average retention time. Fresh medium was perfused in single-pass and the stream leaving the bioreactor Q_{out} was collected as waste until approaching the steady state. When the system reached the steady state, the stream leaving the bioreactor was recycled (Q_r) in order to obtain the accumulation of products. In order to favor the adhesion, the HF membranes were modified by coating with poly-L-lysine ($2\mu\text{g}/\text{cm}^2$) followed by either rat-tail collagen-I (lifetechnologies UK) ($5\mu\text{g}/\text{cm}^2$) or fibronectin ($2\mu\text{g}/\text{cm}^2$).

4.2.2 Cell Culture

Human hepatic mesenchymal stem cells (Sciencell, California, US) were expanded in poly-L-lysine-coated ($2\mu\text{g}/\text{cm}^2$) T-75 flasks in mesenchymal stem cell medium supplemented with FBS 5 % and mesenchymal stem cell growth supplement (Sciencell, California, US) for 5 passages. Cells were incubated at 37°C in a 5% CO_2 ; 20% O_2 atmosphere (v/v) with 95% relative humidity for the whole experiment.

4.2.3 Hepatogenic Differentiation

To determine the conditions that result in a good hepatogenic differentiation, various coatings and differentiation protocols were investigated. In batch conditions, collagen-I and fibronectin coatings as well as no coating were studied to determine the effect of coating on cell differentiation. One protocol for hepatogenic differentiation (Protocol 1) was carried out as described by Lee et al (246). Briefly, cells at 5th passage, at $1.0 \times 10^4/\text{cm}^2$, were serum deprived for 2 days, in Iscove's Modified Dulbecco's Medium (IMDM) Sigma-Aldrich, Milan, Italy) supplemented with 20 ng/mL epidermal growth factor (EGF) and 10 ng/mL basic fibroblast growth factor (bFGF), prior to induction by a 2-step protocol.

Differentiation was induced by treating hHMSCs with Step-1 differentiation medium, consisting of IMDM supplemented with 20 ng/mL hepatocyte growth factor (HGF) and 10 ng/mL bFGF, nicotinamide 0.61 g/L, for 7 days, followed by treatment with step-2 maturation medium, consisting of IMDM supplemented

with 20 ng/mL oncostatin M (OSM), 1 μ mol/L dexamethasone, and 50 mg/mL insulin-transferrin-selenium (ITS+) premix (Sigma-Aldrich). Medium changes were performed twice weekly. All growth factors were purchased from Peprotech (London, UK).

On the other hand, the differentiation protocol (Protocol 2) specifically designed for the hHMSCs involved the seeding of cells at fifth passage at a density of 1×10^4 cells/cm². The two-step protocol was tested only in batch system. First, the cells were treated with Dulbecco's Modified Eagle Medium (DMEM; low glucose) containing 1% insulin-transferrin-selenium (ITS), 10 ng/mL fibroblast growth factor-1 (FGF-1), 10 ng/mL FGF-4, and 20 ng/mL HGF for 5 days. Then they were sequentially treated with the same basal medium containing 100 nM dexamethasone (Sigma-Aldrich, St. Louis, Mo), 10 ng/mL FGF-4, 20 ng/mL HGF, 10 ng/mL OSM, and 0.5% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, Mo) for 20 days.

4.2.4 Cell Morphology

Cell morphology in the HF membrane system was evaluated by light-inverted microscopy, and Confocal Laser Scanning Microscopy (CLSM) analyses. Samples were examined and representative images displaying the cell features were obtained at different culture time.

Cells were fixed with 4% formaldehyde for 20 minutes, followed by permeabilization and blocking in 0.3% Triton-X and 10% fetal bovine serum (FBS) for 1 hour at room temperature. HMSCs were stained for the stem cell markers CD90 and CD44; whereas differentiated cells were stained for both stem cell marker CD90 and hepatic markers α -feto protein (AFP) and albumin (Alb). AFP was detected by using specific goat monoclonal antibodies (Bethyl laboratories), diluted 1:200 in 1% FBS and a CyTM5-conjugated AffiniPure donkey anti-goat IgG (Jackson ImmunoResearch Europe Ltd) were used. Alb and CD44 was visualized by using a specific mouse monoclonal antibody (BD Biosciences) (diluted 1:200 in 1% FBS) raised against the glycoprotein CD31 of human origin and a CyTM3-conjugated AffiniPure donkey anti-mouse IgG (Jackson ImmunoResearch Europe Ltd). CD90 was detected by using specific polyclonal rabbit antibodies (Thermo Fischer) diluted 1:200 in 1% FBS and a CyTM2-conjugated AffiniPure donkey anti-rabbit IgG (Jackson ImmunoResearch Europe Ltd). All primary antibodies were incubated overnight at 4° C; while secondary antibodies were incubated for 1 hours at room temperature. Counterstaining for nuclei was performed with DAPI 0.2 μ g/ml incubated for 20 min. Finally, samples were washed, mounted and viewed with CLSM.

4.2.5 Assay of metabolic functions

Samples of the culture medium were collected and assayed for urea and albumin concentration. The urea concentration was assayed by the enzymatic urease method.

Albumin production in the samples was measured by the immunoenzymatic method (ELISA) using antibodies against human albumin. Ninety-six well plates were coated with 50µg/ml monoclonal anti-albumin antibody (diluted 1:100) and left at room temperature for one hour. This was followed by blocking using 10% BSA solution overnight at 4°C. Afterwards, 100 µl of cell culture supernatant was added to the wells and incubated at room temperature for one hour. Following this step, horseradish peroxidase-conjugated secondary antibodies against albumin (diluted 1:10000) were added for one hour at room temperature. The substrate buffer containing tetramethylbenzidine and H₂O₂ was added for 10 min. The reaction was stopped with 0.18 N H₂SO₄. Absorbance was measured at 450 nm using a Multiskan Ex (Thermo Lab Systems).

4.2.6 Periodic Acid-Schiff (PAS) Stain for Glycogen

Culture dishes containing cells were fixed in 4% formaldehyde, permeabilized with 0.1% Triton X-100 for 10 minutes and were then oxidized in 1% periodic acid (Sigma-Aldrich, Milan, Italy) for 5 minutes, rinsed 3 times in deionized (d)H₂O, treated with Schiff's reagent (Sigma-Aldrich, Milan, Italy) for 15 minutes, and rinsed in dH₂O for 5 to 10 minutes. Samples were counterstained with Mayer's hematoxylin (Sigma-Aldrich, Milan, Italy) for 1 minute and rinsed in dH₂O and assessed under light microscope.

4.2.7 Total RNA Isolation and Reverse-Transcription Polymerase Chain Reaction

RNA was extracted from differentiating cells using Aurum™ Total RNA Mini Kit (Biorad, California, USA) per the manufacturer's instructions. DNase I (Biorad, California, USA) digestion was performed to minimize the possibility of genomic DNA contamination. 150 ng of total RNA was used to synthesize complementary DNA via reverse transcription using iScript™ Reverse Transcription Supermix (Biorad, California, USA) per the manufacturer's instructions. Complementary DNA was amplified using SsoAdvanced™ Universal SYBR® Green Supermix (Biorad, California, USA) at 95°C for 30 seconds for polymerase activation, followed by 40 cycles of 95°C for 15 seconds, and 60°C for 30 seconds for amplification. Real-Time PCR machine MiniOpticon™ (Biorad, California, USA) was utilized. Primers used were bought from Biorad and are listed in Table 4.1.

The 2- $\Delta\Delta Cq$ (Livak) Method was used to relatively quantify the gene expression of the early (AFP) and late (HNF-4 α and ALB) hepatic genes.

$$\Delta\Delta Cq = \Delta Cq(\text{test}) - \Delta Cq(\text{calibrator})$$

$$\Delta Cq(\text{test}) = Cq(\text{target, test}) - Cq(\text{ref, test})$$

$$\Delta Cq(\text{calibrator}) = Cq(\text{target, calibrator}) - Cq(\text{ref, calibrator})$$

Where Cq is the quantification cycle, ref is the reference gene β -actin and hHMSCs on Collagen-I-coated plates were used as a calibrator since Collagen-I is the standard coating used in most differentiation studies as well as in hepatocyte cultures.

Table 4.1. Primers Used for Reverse-Transcription Polymerase Chain Reaction

Primer	Amplicon Context Sequence	Unique Assay ID	Amplicon Length (bp)
ACTB	GTGCTCGATGGGGTACTTCAGGGTGAGGATGCCTC TCTTGCTCTGGGCCTCGTCGCCACATAGGAATCCT TCTGACCCATGCCACCATCA	qHsaCED0036269	62
AFP	GGAACAACCTTGAGGCTGTCATTGCAGATTTCTCAGG CCTGTTGGAGAAATGCTGCCAAGGCCAGGAACAGG AAGTCTGCTTTGCTGAAGAGGGACAAAACTGATTT CAAAAACCTCGTGCTGCTTTGGGAGTTTAAATTAATTC AGGGGAAGAGAAGACAAAACGAGTCTTT	qHsaCID0017919	141
ALB	AGCTGCTGATTTTGTGAAAGTAAGGATGTTTGCAA AAACTATGCTGAGGCAAAGGATGTCTTCCTGGGCAT GTTTTGTATGAATATGCAAGAAGGCATCCTGATTA CTCTGTCGTGCTGCTGCTGAGACTTGCCAAGACA	qHsaCID0017798	112
HNF-4 α	CAATGAGTATGCCTACCTCAAAGCCATCATCTTCTT TGACCCAGATGCCAAGGGGCTGAGCGATCCAGGG AAGATCAAGCGGCTGCGTTCCAGGTGCAGGTGA GCTTGGAGGACTACATCAACGACCGCCAGTATGA	qHsaCID0015879	108

Abbreviations: ACTB, actin beta ; AFP, alpha-fetoprotein ; ALB, albumin ; HNF-4 α , hepatocyte nuclear factor 4 alpha.

4.2.8 Statistical analysis

The statistical significance of the experimental results was calculated using ANOVA test (p <0.05).

4.3 Results

4.3.1 Morphological evaluation of differentiating cells on various coatings

Cells were cultured in batch system on uncoated and coated plates with fibronectin and collagen in order to evaluate the effect of ECM proteins on the hepatogenic differentiation.

Cells cultured using the differentiation (Protocol 2) didn't show signs of morphological differentiation and most of the cultured cells died after 21 days possibly due to DMSO toxicity. Thus, no further analysis was carried out for these cells.

Morphological analysis of the undifferentiated and differentiating human hepatic mesenchymal stem cells (Protocol 1) on various coated and uncoated plates through light microscopy showed morphological changes from an elongated, spindle morphology to a hepatocyte-like highly round or polygonal shape at day 24 of culture (Fig 4.1). In all conditions, the cell number decreased at day 24 compared to the starting undifferentiated seeded hHMSCs, with fibronectin-coated plates showing the lowest cell density. This might be due to the degradation of the fibronectin by enzymes released by the cells.

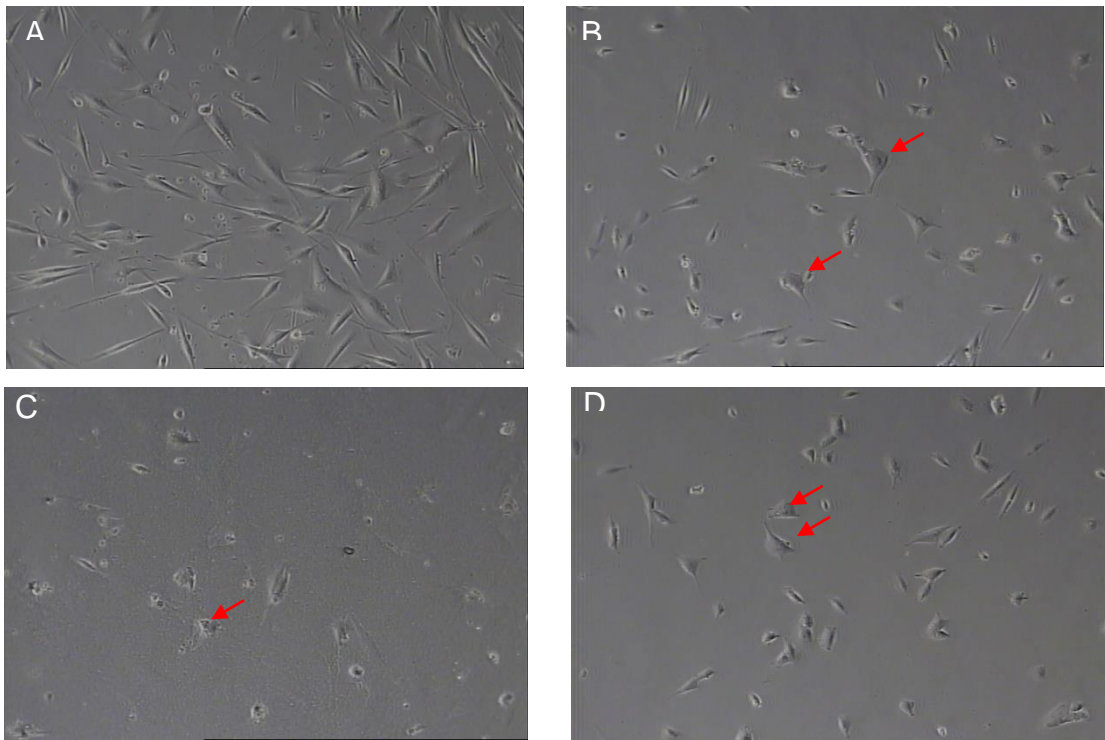


Fig. 4.1 Light Microscope images of A) hHMSCs before differentiation; differentiating cells on B) Collagen-I coated, C) Fibronectin-coated, D) uncoated plates at day 24 of culture. Red arrows point at cells that changed their morphology to adapt a hepatocyte-like morphology. Pictures taken at Ob 10X.

Immunohistochemical staining of mesenchymal, early and late hepatic markers was performed to evaluate the cell differentiation in batch cultures. hHMSCs stained strongly for the mesenchymal marker CD90 and to a lesser extent to CD44, as shown in Fig 4.2. However, they stained negative for hepatic markers AFP and Alb.

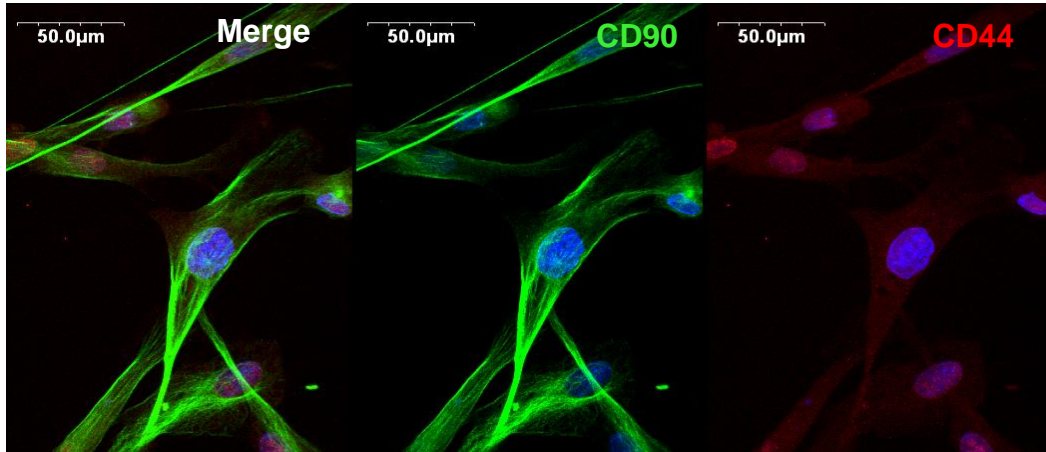
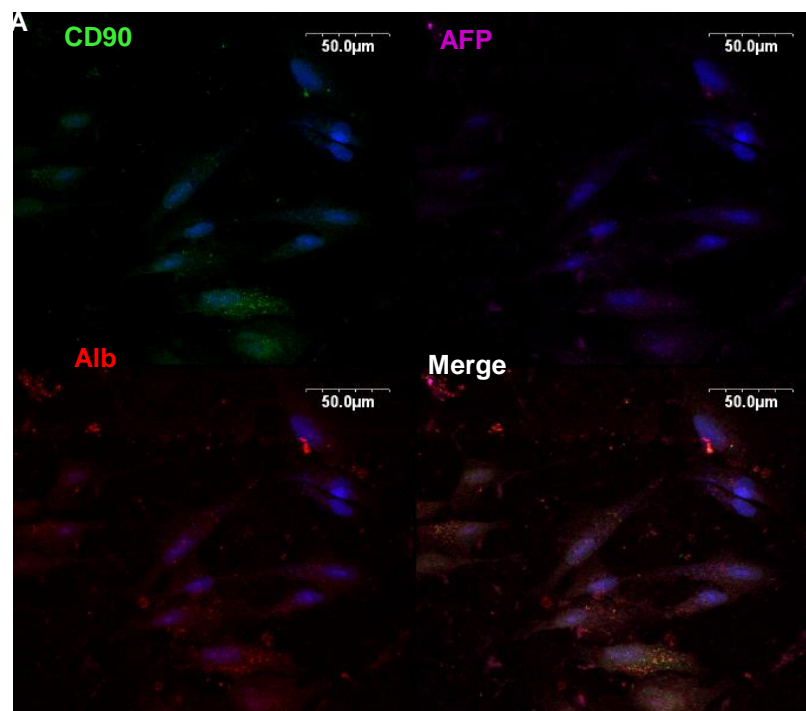


Fig.4.2. LCSM images of human hepatic mesenchymal stem cells in batch culture on poly-L-lysine coated slides prior to differentiation. Cells were stained for the mesenchymal cell markers CD90 (green), CD44 (red) and nuclei (blue).

Differentiating cells in batch systems at day 24 of culture on all coatings displayed a similar trend, as shown in Fig. 4.3. The cells stained very weakly for the mesenchymal cell marker CD90, suggesting that these cells are losing or lost their stemness. On the other hand, the cells stained faintly for early and late hepatic markers, AFP and Alb, respectively.



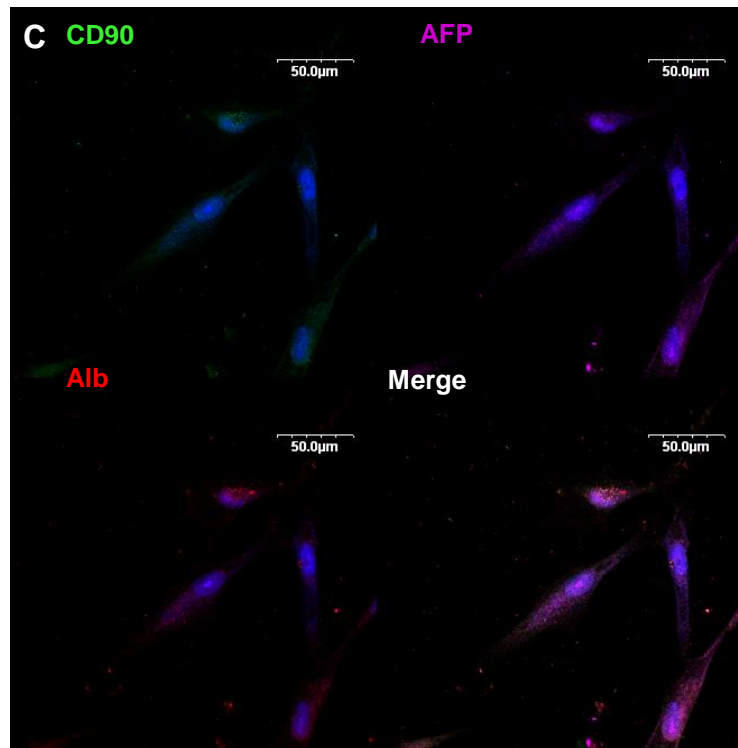
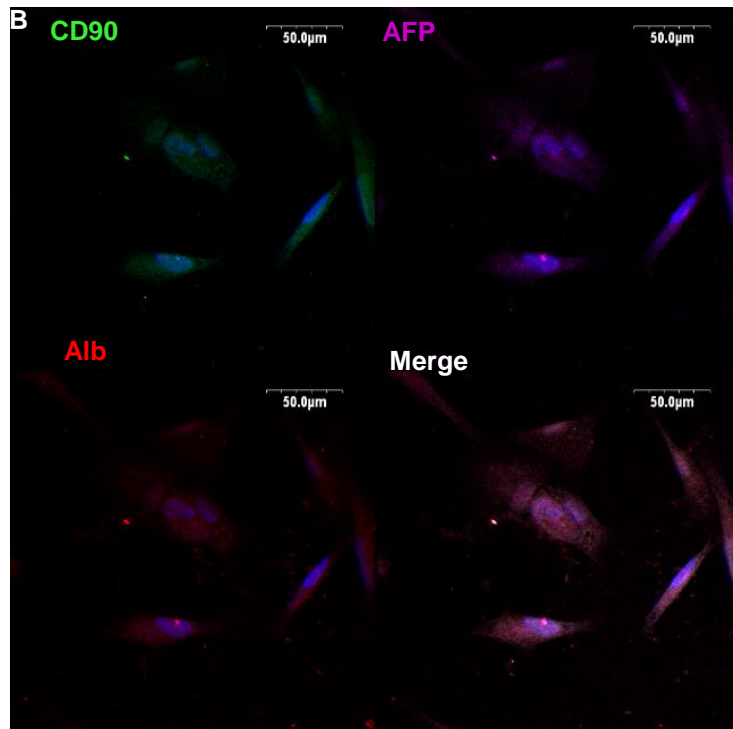


Fig.4.4. LCCM images of differentiating human hepatic mesenchymal stem cells in batch culture on A) Fibronectin-coated, B) Uncoated and C) Collagen-I coated slides at day 24 of culture. Cells were stained for the mesenchymal cell markers CD90 (green), early hepatic marker AFP (red), late hepatic marker Alb (Magenta) and nuclei (blue).

4.3.2 Gene expression profile of treated cells

To determine whether morphologic changes were sustained and associated with the induction of hepatocyte-specific genes, the total RNA was isolated at day 24. Quantitative RT-PCR was used to analyze the expression of early (AFP) and late (ALB and HNF-4 α) genes. Total RNA extracted per million seeded cells was 10 times higher in the case of the bioreactor culture compared to collagen and fibronectin-coated batch cultures; whereas it was 6 times higher than that extracted in the case of uncoated batch cultures.

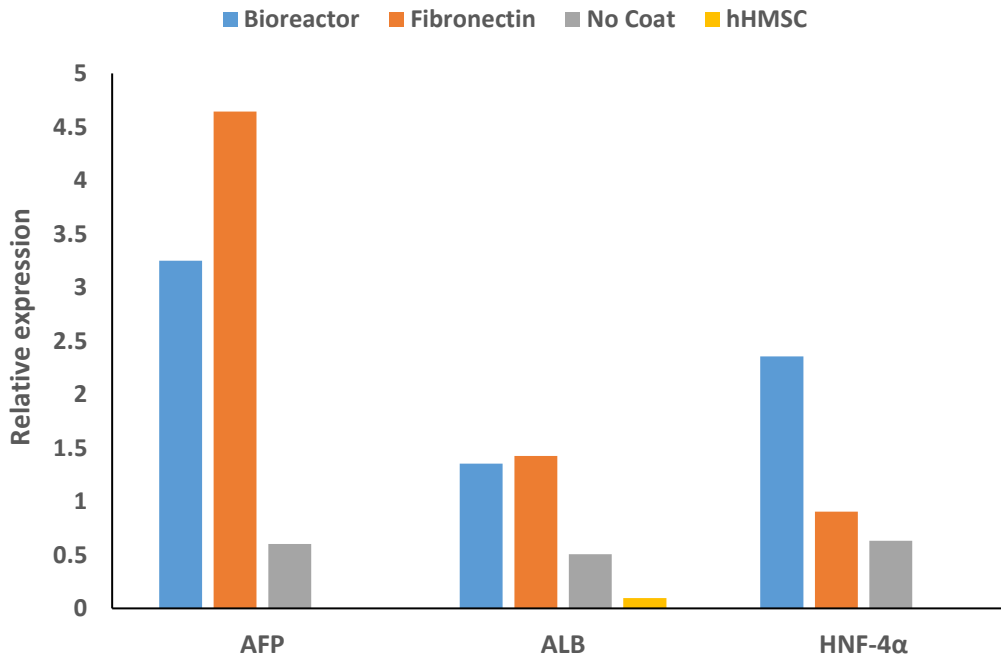


Fig. 4.5. Quantitative RT-PCR analysis of mRNA levels for early (AFP) and late (ALB and HNF-4 α) liver specific markers relative to expression in batch cultures on collagen-I-coated plates.

In all experimental conditions, AFP, ALB and HNF-4 α were expressed at day 24 of culture. However, the levels of expression were different, as shown in fig 4.5. Undifferentiated hHMSCs expressed low levels of AFP and ALB, but no HNF-4 α .

We found highest expression of AFP in case of cell cultured on fibronectin-coated plates; whereas that of HNF-4 α was highest in cells cultured in the bioreactor with respect to the other culture conditions. For albumin gene expression, both cells cultured in the bioreactor and on fibronectin-coated plates expressed ALB at similar levels. However, in both the bioreactor and fibronectin-coated batch cultures, expressed all 3 genes at higher levels compared to that of the Collagen-I-coated batch cultures. On the other hand, hHMSCs in uncoated batch cultures expressed all 3 genes at a lower level relative to their expression in Collagen-I-coated batch cultures.

4.3.3 Hepatocyte specific functions

Three different assays to determine whether these hepatocyte-like cells also had functional features related to hepatocyte attributes were used; namely urea synthesis, albumin production and glycogen storage.

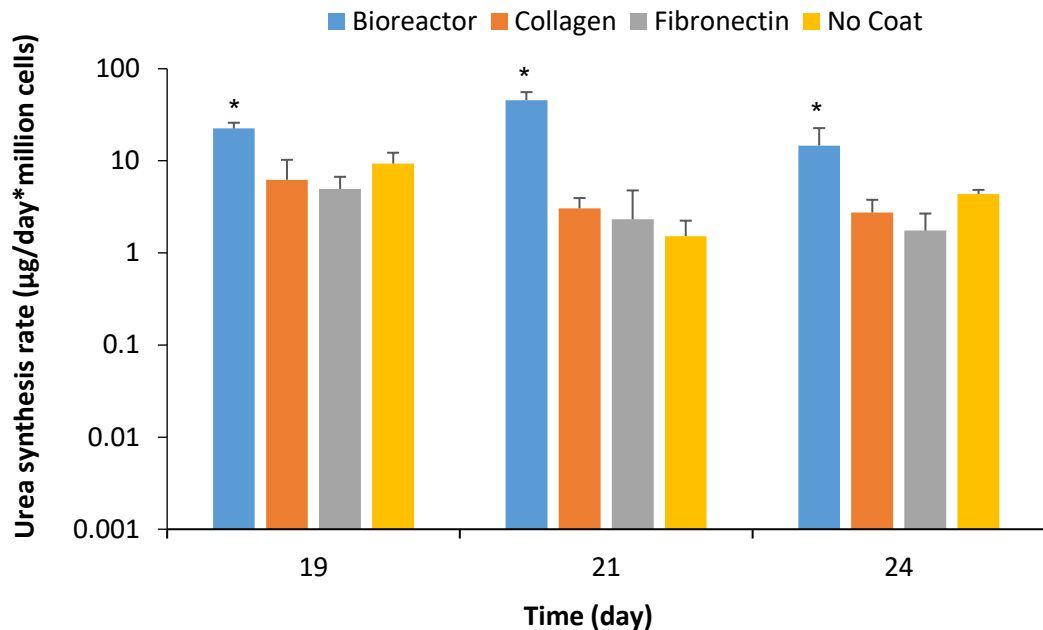


Fig. 4.6. Urea synthesis rate of differentiated hMSCs in hollow fiber membrane bioreactor and batch cultures on collagen-I coated, fibronectin-coated and uncoated plates. The values are the mean of three experiments \pm standard deviation. Data statistically significant $p < 0.05$: (*) vs. batch system at same day.

Differentiated hMSCs synthesized urea after 19 days in culture under all experimental conditions, with the bioreactor culture showing significantly higher synthesis rates compared to the batch cultures, as shown in Fig 4.6.

All batch cultures showed almost similar urea synthesis rates ranging from approximately 1.7 ± 0.3 to 6.1 ± 2.9 $\mu\text{g}/\text{d} \cdot \text{million cells}$, while the urea synthesis rates for bioreactor culture ranged from approximately 14.7 ± 7 to 45.7 ± 10 $\mu\text{g}/\text{d} \cdot \text{million cells}$.

As shown in Fig. 4.7, differentiated hMSCs on uncoated plates synthesized the highest amount of albumin among the various batch cultures with rates ranging from 1.6 ± 0.4 $\text{ng}/\text{d} \cdot \text{million cells}$ to 4 ± 0.4 $\text{ng}/\text{d} \cdot \text{million cells}$. On the other hand, cells on fibronectin-coated and collagen-coated plates synthesized albumin at similar rates. Albumin synthesis rates of the bioreactor were much higher than those of the batch cultures with an average of 59.1 ± 7.7 $\text{ng}/\text{d} \cdot \text{million cells}$.

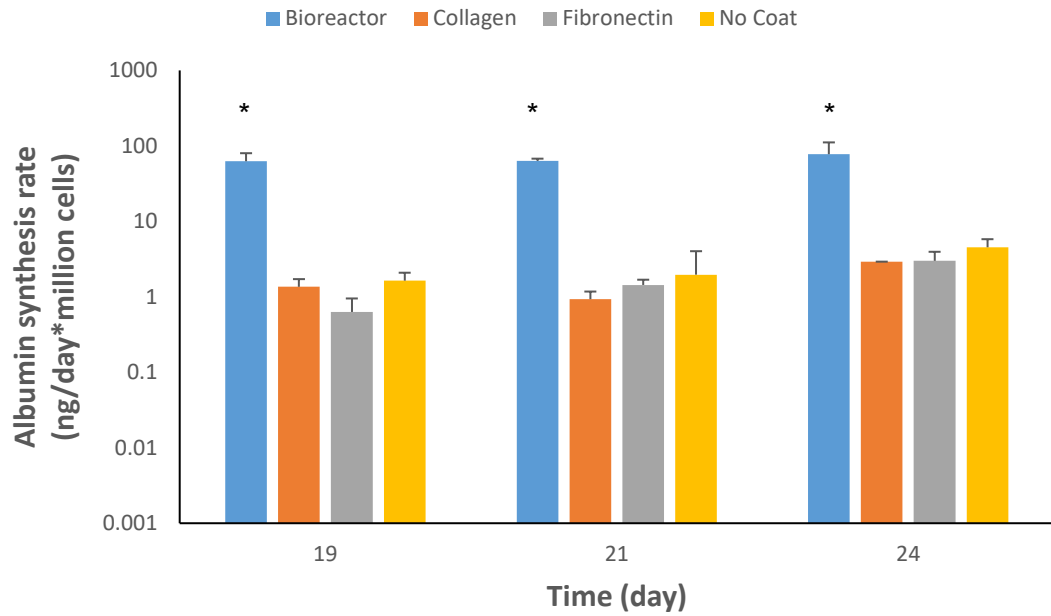


Fig. 4.7. Albumin synthesis rate of differentiated hMSCs in hollow fiber membrane bioreactor and batch cultures on collagen-I coated, fibronectin-coated and uncoated plates. The values are the mean of three experiments \pm standard deviation. Data statistically significant $p < 0.05$: (*) vs. batch system at same day.

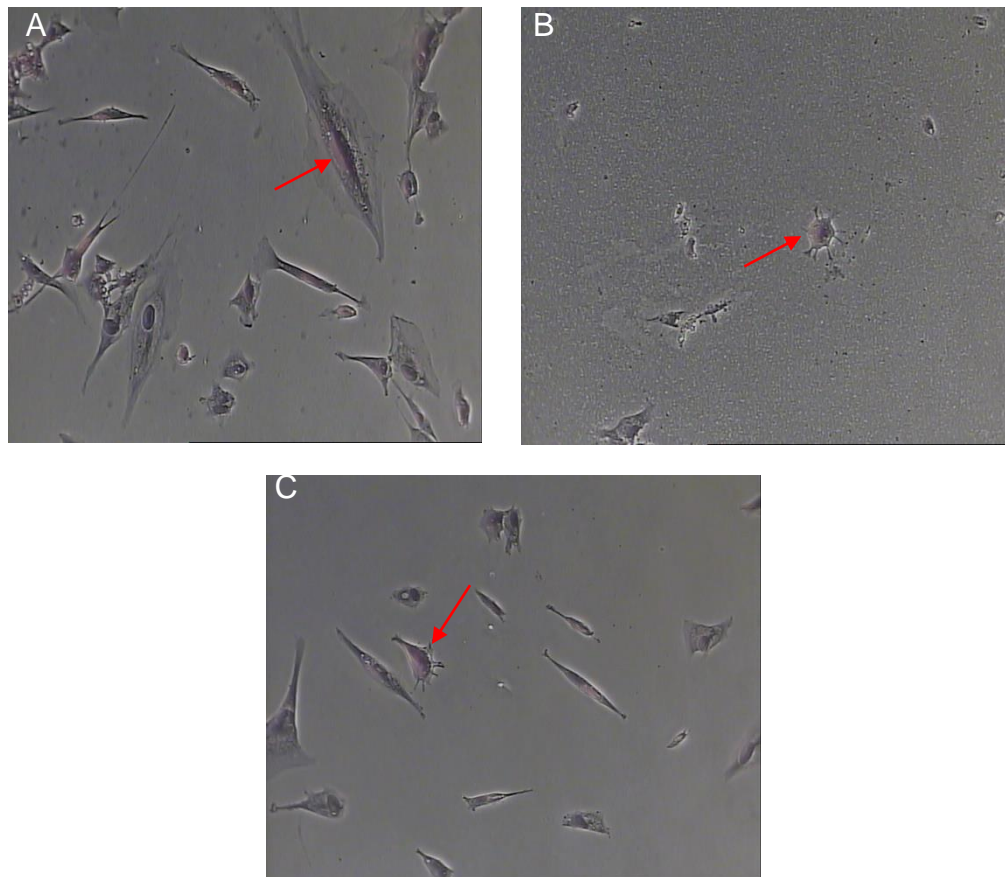


Fig. 4.8. Glycogen storage depicted by periodic acid-Schiff (PAS) staining showed few positive cells after 24 days on A) Collagen-I-coated, B) Fibronectin-coated, C) Uncoated plates as indicated by the red arrows. Pictures taken at Ob 20X.

The presence of stored glycogen, as determined by PAS staining, was observed in few cells at day 24 in batch cultures, as shown in fig 4.8.

4.4 Discussion

The regenerative capacity of the liver after partial hepatectomy or chemical injury is well known (249). However, the cell types involved in hepatic regeneration are still undefined, and a contribution of both mature hepatocytes and resident stem cells has been suggested. Evidence from several studies indicates the presence of resident stem cells in the adult liver (250). In this work, the recently discovered mesenchymal stem cell population residing in the human liver (hHMSC) were used. Differentiation conditions by using different protocol and different coatings were studied in batch systems to determine the optimal conditions to employ in the bioreactor.

Using the protocol described for bone marrow-derived stem cell differentiation (protocol 1), cells cultured on both coated and uncoated plates underwent differentiation. In the initial induction step, MSCs are induced into endodermal cells by epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). Both bFGF and EGF stimulates proliferation of MSCs (251, 252). bFGF plays a pivotal role during the initial stage of endodermal patterning (253). FGF, HGF, nicotinamide (NTA) and insulin-transferrin-selenium (ITS) are commonly added to cultures to trigger cell differentiation. HGF is a pleiotropic cytokine of mesenchymal origin involved in the regulation of proliferation, differentiation, and chemotactic migration of MSCs (244). Adipose stem cells cultured on HGF/collagen I spots for 2 weeks were found to exhibit an increased expression of hepatocyte-specific genes, indicating induction of hepatic differentiation by HGF (254). Interestingly, Forte et al. (255) showed that long-term exposure of MSCs to HGF resulted in cytoskeletal rearrangement, cell migration, and marked inhibition of proliferation. The differentiation efficacy of various cytokines, including HGF, ITS, dexamethasone and NTA, was studied, and HGF and NTA were reported to be the most potent inducers (256). HGF was shown to promote a mid/late hepatic phenotype but failed to induce functional hepatocyte maturation (257). To induce further maturation, oncostatin M (OSM) and dexamethasone are required, together with the addition of FGF, ITS, and HGF. OSM is a member of the IL-6 subfamily that plays an important role in progression from hepatocyte development to liver maturation (258). Dexamethasone induces the expression of HNF-4 α , which is essential for hepatocyte differentiation (259).

Regarding the effect of coating on the differentiation potential, liver-specific functions, namely urea and albumin synthesis, were not significantly different for cell on coated and uncoated plates. However, fibronectin-coated plates showed fewer number of cells and signs of coating degradation which would lead to cell loss if used in the bioreactor. Also, the PES fibers used in the bioreactor are not cell adhesive and hence can't be left uncoated; otherwise cells would not attach to the fibers and die. Conversely, collagen coating retained its integrity till the end of the experiment.

On the other hand, the protocol described for the differentiation of hMSCs (protocol 2) did not lead to cell differentiation; rather, cells died in culture probably due to the use of DMSO. Even though DMSO was described in literature to enhance maturation of hepatocyte-like cells, it was used in a lower concentration of only 0.1% instead of 0.5% in the aforementioned protocol (260).

Based on these findings, protocol 1 and collagen coating were utilized for the HFMBR culture. The constant perfusion of medium in the bioreactor ensures not only a constant supply of nutrients and growth factors to the cells, but also the removal of metabolic products. This allows for a uniform microenvironment leading to a better hepatogenic differentiation of hMSCs in the bioreactor compared to the batch cultures as shown by the higher gene expression of adult hepatocyte marker HNF-4 α as well as the significantly higher liver-specific functions, namely urea and albumin synthesis rates. Albumin is the major serum protein produced by mature hepatocytes thus its expression is a sign of hepatic maturation. In the present study, cells cultured in bioreactors under perfusion conditions exhibited 15-fold higher albumin secretion than cells grown under conventional static culture conditions. Since albumin secretion of the liver is regulated by an intrinsic feed-back mechanism (261), a permanent removal of albumin by perfusion with constant feed is likely to induce increased albumin production and consequently contributes to hepatic maturation.

4.5 Conclusions

In conclusion, culture conditions of human hepatic mesenchymal stem cells were established in batch system regarding the coating and the differentiation protocols. Thereafter, the optimized conditions were utilized for the bioreactor culture. The gene expression of AFP, Alb and HFN-4 α demonstrated higher hepatic commitment of the cells cultured into the bioreactor in comparison with other batch culture conditions. This was corroborated by the performance of urea synthesis and albumin secretion after 24 days of culture. The cell differentiation depends on controlling key process variables: nutrient and metabolite

concentrations, growth factor compositions, and physiological parameters. The crossed HF membrane bioreactor creates a homogeneous environment for cell culture in which the concentrations of nutrients metabolites and growth factors are monitored and controlled and differentiation signals are provided to the cells. Selective exchange of gases and metabolites through the selective HF membranes ensured a microenvironment adequate for the hepatogenic differentiation and the performance of liver specific functions. These promising results pave the way for further studies that might one day result in fully differentiated functional hepatocytes, which in turn would solve the problem of insufficiency of primary human hepatocytes for use in BAL devices.

General Conclusion and Future Perspectives

In this work, lab-scale bioreactor cultures were established for the long-term maintenance of hepatocyte viability and functionality *in vitro* in various setups (microtissue spheroids and organotypic cultures). In addition, hepatogenic mesenchymal stem cell differentiation was established and optimized. The resulting conclusions and perspectives are detailed below:

1- An important aspect of this work is using human primary hepatocytes rather than animal hepatocytes or cell lines. Even though human hepatocytes pose a financial hurdle, they are the only cell type that recapitulates the *in vivo* behavior inside the human body and thus is the optimal cell source for a bio-artificial liver device for use as temporary support for liver failure patients or for drug screening. Various cultures using primary human hepatocytes were described in literature, the most famous of which and considered the gold standard is collagen sandwich, in which hepatocytes are cultured as single layers of cells between two layers of collagen. However, this and other models lack key aspects for a model that closely recapitulates the *in vivo* situation which are a) 3D cultures in which cells are surrounded with other cells from all directions which is crucial for the cell polarity, which in turn affects cell function and viability, b) co-culture of hepatocytes with other non-parenchymal cells of the liver that have been reported to play an important role in hepatocyte survival and enhanced functions. Thus, in this work human hepatocytes were cultured both alone in spheroidal configuration and as single cells in co-culture with sinusoidal endothelial and stellate cells.

2- Another important aspect of the work is using a crossed hollow-fiber membrane bioreactor (HFMBR), which, unlike other bioreactor types, protects cells from shear stress while allowing for unrestricted flow (depending on the properties of the used fibers) of nutrients into and catabolites out of the bioreactor. Moreover, the hollow-fiber membranes allow cell anchorage, their proliferation, migration and reassembly. Furthermore, this bioreactor can be easily scaled-up from lab to clinical-scale. However, upon scaling-up issues such as adequate oxygenation of the cultured cells might arise, requiring certain modifications of the setup by adding an oxygenator to the medium prior to the inlet or adding oxygenating tubes inside the bioreactor.

3- 3D cultures of human hepatocytes spheroids cultured into the HFMBR were maintained functional (assessed in terms of urea and albumin synthesis, and diazepam metabolism) and viable for almost a month. 2D cell cultures allow cell maintenance for approximately 3-7 days. The 3D architecture supports the

functional cell polarization mimicking, to an extent, the *in vivo* situation in which hepatocytes are surrounded by other cells. The cultured spheroids were found to “move” on the fibers to fuse with other spheroids forming larger microtissue-like structures that shrink with time due to the formation of tight junctions between the cells. The liver microtissue spheroids acquire the cytoarchitecture of the *in vivo* tissue. Additionally, they maintained their liver-specific functions and biotransformation for extended periods.

4- Even though 3D cultures represent an important improvement over the conventional 2D cultures, they lack an important key component that is deemed vital for hepatocyte functionality and viability, which is the cell-cell interaction with the other liver non-parenchymal cells. Thus, organotypic liver cultures were developed via a novel designed approach that mimics the *in vivo* cell layers. In this work, human sinusoidal endothelial cells were seeded first followed by stellate cells and finally, human hepatocytes were seeded to obtain a layered culture with sinusoidal endothelial cells in contact with the hollow fibers (that represent the blood vessels in the body) followed by stellate then hepatocytes. As the culture progressed, cell aggregates formed that increased in size reaching 1mm organoids at the end of the culture. Upon examination of the organoids, they were found to contain capillary-like structures at the periphery and hepatocytes in the middle surrounded by stellate cells; suggesting a re-arrangement of the cultured cells with time. Thus, unlike other bioreactors in which cells are encapsulated and can't re-arrange or fuse, the HFMBR allows for cell attachment and rearrangement in a way similar to that *in vivo*. Cultures lasted for 28 days through which constant monitoring of the liver-specific functions, namely urea, albumin synthesis and diazepam metabolism, was carried out in addition to the dissolved oxygen concentration measurements to ensure the adequacy of the oxygen supply to the cells in order to avoid oxygen limitation and subsequent cell death. The HFMBR was found to maintain a stable and sufficient oxygen supply to the cells, unlike the batch system in which the organotypic cultures suffered significant oxygen limitation.

5- Primary human hepatocytes represent the best cell source for a BAL device; however, due to their scarcity and expensive price an alternative cell source has to be considered for a BAL device to be applicable in a clinical setting or even for use in pharmaceuticals toxicity testing. In this work, mesenchymal stem cells of liver origin have been chosen due to their prior commitment to hepatogenic differentiation in contrast to those from other sources (eg. Fat, Bone Marrow or umbilical cord- derived stem cells). Various conditions (differentiation protocols and coating) were tested into batch cultures, in order to choose the conditions that result in best results. Afterwards, the optimized conditions were used in HFMBR to study the effect of having a homogenous microenvironment on the

hepatogenic differentiation of cells. Indeed, the controlled homogenous microenvironment resulted in better differentiation of the cells compared to the batch cultures. Albeit promising, these results are not yet optimal. Improvements regarding the differentiation protocol including the use of different growth factors are required to achieve enhanced differentiated functions to enable its use as an alternative cell source in a BAL device.

To sum up, in this work culture conditions for 3D cultures (microtissue spheroids) and organotypic cultures (co-culture of hepatocytes and non-parenchymal cells) were optimized allowing for maintenance of functional human primary hepatocytes for 24 and 28 days, respectively. Further optimization is needed to allow for even longer culture. In addition, liver-derived mesenchymal stem cells were successfully differentiated into hepatocyte-like cells after 24 days in culture. This prototype lab-scale bioreactor has many possible applications ranging from basic studies of liver diseases to long-term toxicity studies. Moreover, it can be scaled-up to a clinical scale to be used as BAL device for liver failure patients.

References

1. Ling Q, Xu X, Wei Q, Liu X, Guo H, Zhuang L, et al. Downgrading MELD improves the outcomes after liver transplantation in patients with acute-on-chronic hepatitis B liver failure. *PLoS One*. 2012;7(1):e30322.
2. Bosch FX, Ribes J, Díaz M, Cléries R. Primary liver cancer: worldwide incidence and trends. *Gastroenterology*. 2004;127(5):S5-S16.
3. Jemal A, Center MM, DeSantis C, Ward EM. Global patterns of cancer incidence and mortality rates and trends. *Cancer Epidemiology Biomarkers & Prevention*. 2010;19(8):1893-907.
4. Clavien P-A, Petrowsky H, DeOliveira ML, Graf R. Strategies for safer liver surgery and partial liver transplantation. *New Engl J Med*. 2007;356(15):1545-59.
5. Liou IW, Larson AM, editors. Role of liver transplantation in acute liver failure. *Seminars in liver disease*; 2008: © Thieme Medical Publishers.
6. Chan AC, Fan ST, Lo CM, Liu CL, Chan SC, Ng KK, et al. Liver transplantation for acute-on-chronic liver failure. *Hepatology international*. 2009;3(4):571-81.
7. Strain AJ, Neuberger JM. A bioartificial liver--state of the art. *Science*. 2002;295(5557):1005-9.
8. Schuster D, Laggner C, Langer T. Why drugs fail-a study on side effects in new chemical entities. *Curr Pharm Des*. 2005;11(27):3545-59.
9. Wilke RA, Lin DW, Roden DM, Watkins PB, Flockhart D, Zineh I, et al. Identifying genetic risk factors for serious adverse drug reactions: current progress and challenges. *Nature reviews Drug discovery*. 2007;6(11):904-16.
10. Zguris JC, Itle LJ, Hayes D, Pishko MV. Microreactor microfluidic systems with human microsomes and hepatocytes for use in metabolite studies. *Biomed Microdevices*. 2005;7(2):117-25.
11. Olinga P, Schuppan D. Precision-cut liver slices: a tool to model the liver ex vivo. *Journal of hepatology*. 2013;58(6):1252-3.
12. Gebhardt R, Hengstler JG, Müller D, Glöckner R, Buenning P, Laube B, et al. New hepatocyte in vitro systems for drug metabolism: metabolic capacity and recommendations for application in basic research and drug development, standard operation procedures. *Drug Metab Rev*. 2003;35(2-3):145-213.
13. Sivaraman A, Leach J, Townsend S, Iida T, Hogan B, Stolz DB, et al. A microscale in vitro physiological model of the liver: predictive screens for drug metabolism and enzyme induction. *Curr Drug Metab*. 2005;6(6):569-91.
14. Dancygier H. *Clinical hepatology: Principles and practice of hepatobiliary diseases*: Springer Science & Business Media; 2009.
15. Mescher AL. *Junqueira's basic histology: text and atlas*: McGraw-hill; 2013.
16. Gebhardt R. Metabolic zonation of the liver: regulation and implications for liver function. *Pharmacol Ther*. 1992;53(3):275-354.
17. Gooding P, Chayen J, Sawyer B, Slater T. Cytochrome P-450 distribution in rat liver and the effect of sodium phenobarbitone administration. *Chem-Biol Interact*. 1978;20(3):299-310.
18. Bengtsson G, Julkunen A, Penttilä K, Lindros K. Effect of phenobarbital on the distribution of drug metabolizing enzymes between periportal and perivenous rat hepatocytes prepared by digitonin-collagenase liver perfusion. *J Pharmacol Exp Ther*. 1987;240(2):663-7.
19. Li Z, Diehl A. Innate immunity in the liver. *Current opinion in gastroenterology*. 2003;19(6):565-71.
20. Katz N, Teutsch HF, Jungermann K, Sasse D. Heterogeneous reciprocal localization of fructose-1, 6-bis-phosphatase and of glucokinase in microdissected periportal and perivenous rat liver tissue. *FEBS Lett*. 1977;83(2):272-6.
21. Häussinger D, Lamers W, Moorman A. Hepatocyte heterogeneity in the metabolism of amino acids and ammonia. *Enzyme*. 1991;46(1-3):72-93.

22. Jungermann K, Keitzmann T. Zonation of parenchymal and nonparenchymal metabolism in liver. *Annu Rev Nutr.* 1996;16(1):179-203.
23. Braeuning A, Ittrich C, Köhle C, Hailfinger S, Bonin M, Buchmann A, et al. Differential gene expression in periportal and perivenous mouse hepatocytes. *FEBS J.* 2006;273(22):5051-61.
24. Cagle PT, Monga SP. *Molecular pathology of liver diseases*: Springer Science & Business Media; 2010.
25. Carpentier B, Gautier A, Legallais C. Artificial and bioartificial liver devices: present and future. *Gut.* 2009;58(12):1690-702.
26. Rademacher S, Oppert M, Jörres A. Artificial extracorporeal liver support therapy in patients with severe liver failure. *Expert review of gastroenterology & hepatology.* 2014.
27. Taub R. Liver regeneration: from myth to mechanism. *Nature reviews Molecular cell biology.* 2004;5(10):836-47.
28. Michalopoulos GK, DeFrances MC. Liver regeneration. *Science.* 1997;276(5309):60-6.
29. Arias IM, Che M, Gatmaitan Z, Leveille C, Nishida T, St Pierre M. The biology of the bile canaliculus, 1993. *Hepatology.* 1993;17(2):318-29.
30. Wanson J, Bernaert D, May C. Morphology and functional properties of isolated and cultured hepatocytes. *Progress in Liver diseases.* 1978;6:1-22.
31. Celton-Morizur S, Merlen G, Couton D, Desdouets C. Polyploidy and liver proliferation: central role of insulin signaling. *Cell Cycle.* 2010;9(3):460-6.
32. Bertolino P, Trescol-Biémont MC, Rabourdin-Combe C. Hepatocytes induce functional activation of naive CD8⁺ T lymphocytes but fail to promote survival. *Eur J Immunol.* 1998;28(1):221-36.
33. Racanelli V, Rehmann B. The liver as an immunological organ. *Hepatology.* 2006;43(S1).
34. Overturf K, Al-Dhalimy M, Finegold M, Grompe M. The repopulation potential of hepatocyte populations differing in size and prior mitotic expansion. *The American journal of pathology.* 1999;155(6):2135-43.
35. Lowes KN, Croager EJ, Olynyk JK, Abraham LJ, Yeoh GC. Oval cell-mediated liver regeneration: Role of cytokines and growth factors. *Journal of gastroenterology and hepatology.* 2003;18(1):4-12.
36. Friedman SL. Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. *Physiol Rev.* 2008;88(1):125-72.
37. Bilzer M, Roggel F, Gerbes AL. Role of Kupffer cells in host defense and liver disease. *Liver International.* 2006;26(10):1175-86.
38. Knolle PA, Limmer A. Control of immune responses by scavenger liver endothelial cells. *Swiss medical weekly.* 2003;133(37/38):501-6.
39. Limmer A, Ohl J, Wingender G, Berg M, Jüngerkes F, Schumak B, et al. Cross-presentation of oral antigens by liver sinusoidal endothelial cells leads to CD8 T cell tolerance. *Eur J Immunol.* 2005;35(10):2970-81.
40. Wake K. "Sternzellen" in the liver: perisinusoidal cells with special reference to storage of vitamin A. *American Journal of Anatomy.* 1971;132(4):429-61.
41. Gutiérrez-Ruiz M, Gómez-Quiroz LE. Liver fibrosis: searching for cell model answers. *Liver International.* 2007;27(4):434-9.
42. Paik YH, Schwabe RF, Bataller R, Russo MP, Jobin C, Brenner DA. Toll-like receptor 4 mediates inflammatory signaling by bacterial lipopolysaccharide in human hepatic stellate cells. *Hepatology.* 2003;37(5):1043-55.
43. Winau F, Hegasy G, Weiskirchen R, Weber S, Cassan C, Sieling PA, et al. Ito cells are liver-resident antigen-presenting cells for activating T cell responses. *Immunity.* 2007;26(1):117-29.
44. Viñas O, Bataller R, Sancho-Bru P, Ginès P, Berenguer C, Enrich C, et al. Human hepatic stellate cells show features of antigen-presenting cells and stimulate lymphocyte proliferation. *Hepatology.* 2003;38(4):919-29.

45. Senoo H, Kojima N, Sato M. Vitamin A-storing cells (stellate cells). *Vitamins & Hormones*. 2007;75:131-59.
46. Wake K, Decker K, Kirn A, Knook D, McCuskey R, Bouwens L, et al. Cell biology and kinetics of Kupffer cells in the liver. *Int Rev Cytol*. 1989;118:173-229.
47. Gale RP, Sparkes RS, Golde DW. Bone marrow origin of hepatic macrophages (Kupffer cells) in humans. *Science*. 1978;201(4359):937-8.
48. Wheeler HO, Ramos OL. Determinants of the flow and composition of bile in the unanesthetized dog during constant infusions of sodium taurocholate. *J Clin Invest*. 1960;39(1):161.
49. Alpini G, McGill JM, LaRusso NF. The pathobiology of biliary epithelia. *Hepatology*. 2002;35(5):1256-68.
50. Padda MS, Sanchez M, Akhtar AJ, Boyer JL. Drug-induced cholestasis. *Hepatology*. 2011;53(4):1377-87.
51. Jalan R, Sen S, Williams R. Prospects for extracorporeal liver support. *Gut*. 2004;53(6):890-8.
52. Struecker B, Raschzok N, Sauer IM. Liver support strategies: cutting-edge technologies. *Nature Reviews Gastroenterology & Hepatology*. 2014;11(3):166-76.
53. Phua J, Lee KH. Liver support devices. *Current opinion in critical care*. 2008;14(2):208-15.
54. Lanza R, Langer R, Vacanti JP. *Principles of tissue engineering*: Academic press; 2011.
55. Khetani SR, Berger DR, Ballinger KR, Davidson MD, Lin C, Ware BR. Microengineered liver tissues for drug testing. *Journal of laboratory automation*. 2015;20(3):216-50.
56. Ebrahimkhani MR, Neiman JAS, Raredon MSB, Hughes DJ, Griffith LG. Bioreactor technologies to support liver function in vitro. *Adv Drug Del Rev*. 2014;69:132-57.
57. Allen JW, Hassanein T, Bhatia SN. Advances in bioartificial liver devices. *Hepatology*. 2001;34(3):447-55.
58. Sheridan SD, Gil S, Wilgo M, Pitt A. Microporous membrane growth substrates for embryonic stem cell culture and differentiation. *Methods in cell biology*. 2008;86:29-57.
59. Salerno S, Campana C, Morelli S, Drioli E, De Bartolo L. Human hepatocytes and endothelial cells in organotypic membrane systems. *Biomaterials*. 2011;32(34):8848-59.
60. Dunn J, Tompkins RG, Yarmush ML. Hepatocytes in collagen sandwich: evidence for transcriptional and translational regulation. *J Cell Biol*. 1992;116(4):1043-53.
61. Théry M. Micropatterning as a tool to decipher cell morphogenesis and functions. *J Cell Sci*. 2010;123(24):4201-13.
62. Park J, Berthiaume F, Toner M, Yarmush ML, Tilles AW. Microfabricated grooved substrates as platforms for bioartificial liver reactors. *Biotechnol Bioeng*. 2005;90(5):632-44.
63. Diban N, Stamatialis D. Polymeric hollow fiber membranes for bioartificial organs and tissue engineering applications. *J Chem Technol Biotechnol*. 2014;89(5):633-43.
64. Yu Y, Fisher JE, Lillegard JB, Rodysill B, Amiot B, Nyberg SL. Cell therapies for liver diseases. *Liver transplantation*. 2012;18(1):9-21.
65. Tharakan JP, Chau PC. A radial flow hollow fiber bioreactor for the large-scale culture of mammalian cells. *Biotechnol Bioeng*. 1986;28(3):329-42.
66. Wung N, Acott SM, Tosh D, Ellis MJ. Hollow fibre membrane bioreactors for tissue engineering applications. *Biotechnol Lett*. 2014;36(12):2357-66.
67. De Bartolo L, Morelli S, Rende M, Campana C, Salerno S, Quintiero N, et al. Human hepatocyte morphology and functions in a multibore fiber bioreactor. *Macromol Biosci*. 2007;7(5):671-80.
68. Hoesli CA, Luu M, Piret JM. A novel alginate hollow fiber bioreactor process for cellular therapy applications. *Biotechnol Prog*. 2009;25(6):1740-51.
69. Hilal-Alnaqbi A, Mourad A-HI, Yousef BF, Gaylor JD. Experimental evaluation and theoretical modeling of oxygen transfer rate for the newly developed hollow fiber bioreactor with three compartments. *Bio-Med Mater Eng*. 2013;23(5):387-403.

70. Morelli S, Salerno S, Piscioneri A, Campana C, Drioli E, Bartolo LD. Membrane bioreactors for regenerative medicine: an example of the bioartificial liver. *Asia-Pacific Journal of Chemical Engineering*. 2010;5(1):146-59.
71. Yang K-S, Guo X, Meng W, Hyun J-Y, Kang I-K, Kim YI. Behavior of hepatocytes inoculated in gelatin-immobilized polyurethane foam. *Macromolecular research*. 2003;11(6):488-94.
72. Kan P, Miyoshi H, Yanagi K, Ohshima N. Effects of shear stress on metabolic function of the co-culture system of hepatocyte/nonparenchymal cells for a bioartificial liver. *ASAIO journal*. 1998;44(5):M441-M4.
73. Kinasiwicz A, Gautier A, Lewinska D, Bukowski J, Legallais C, Weryński A, editors. Culture of C3A cells in alginate beads for fluidized bed bioartificial liver. *Transplantation proceedings*; 2007: Elsevier.
74. Kanai H, Marushima H, Kimura N, Iwaki T, Saito M, Maehashi H, et al. Extracorporeal Bioartificial Liver Using the Radial-flow Bioreactor in Treatment of Fatal Experimental Hepatic Encephalopathy. *Artif Organs*. 2007;31(2):148-51.
75. Naruse K, Sakai Y, Nagashima I, Jiang G, Suzuki M, Muto T. Comparisons of porcine hepatocyte spheroids and single hepatocytes in the non-woven fabric bioartificial liver module. *The International journal of artificial organs*. 1996;19(10):605-9.
76. Uchino J, Tsuburaya T, Kumagai F, Hase T, Hamada T, Komai T, et al. A hybrid bioartificial liver composed of multiplated hepatocyte monolayers. *ASAIO transactions/American Society for Artificial Internal Organs*. 1987;34(4):972-7.
77. Yanagi K, Miyoshi H, Ohshima N. Improvement of metabolic performance of hepatocytes cultured in vitro in a packed-bed reactor for use as a bioartificial liver. *ASAIO journal*. 1998;44(5):M436-M40.
78. Doran PM. *Bioprocess engineering principles*: Academic press; 1995.
79. Gion T, Shimada M, Shirabe K, Nakazawa K, Ijima H, Matsushita T, et al. Evaluation of a hybrid artificial liver using a polyurethane foam packed-bed culture system in dogs. *J Surg Res*. 1999;82(2):131-6.
80. Yanagi K, Ookawa K, Mizuno S, Ohshima N. Performance of a new hybrid artificial liver support system using hepatocytes entrapped within a hydrogel. *ASAIO Journal*. 1989;35(3):570-1.
81. Doré E, Legallais C. A new concept of bioartificial liver based on a fluidized bed bioreactor. *Therapeutic Apheresis*. 1999;3(3):264-7.
82. Wells G, Fisher M, Sefton M. Microencapsulation of viable hepatocytes in HEMA-MMA microcapsules: a preliminary study. *Biomaterials*. 1993;14(8):615-20.
83. Dixit V, Gitnick G. The bioartificial liver: state-of-the-art. *European Journal of Surgery*. 1998;164(S12):71-6.
84. Takabatake H, Koide N, Tsuji T. Encapsulated multicellular spheroids of rat hepatocytes produce albumin and urea in a spouted bed circulating culture system. *Artif Organs*. 1991;15(6):474-80.
85. Ambrosino G, Basso SM, Varotto S, Zardi E, Picardi A, D'Amico DF. Isolated hepatocytes versus hepatocyte spheroids: in vitro culture of rat hepatocytes. *Cell transplantation*. 2005;14(6):397-401.
86. Kim L, Toh Y-C, Voldman J, Yu H. A practical guide to microfluidic perfusion culture of adherent mammalian cells. *Lab on a Chip*. 2007;7(6):681-94.
87. Lee PJ, Hung PJ, Lee LP. An artificial liver sinusoid with a microfluidic endothelial-like barrier for primary hepatocyte culture. *Biotechnol Bioeng*. 2007;97(5):1340-6.
88. Leclerc E, Sakai Y, Fujii T. Microfluidic PDMS (polydimethylsiloxane) bioreactor for large-scale culture of hepatocytes. *Biotechnol Prog*. 2004;20(3):750-5.
89. Pang Y, Horimoto Y, Sutoko S, Montagne K, Shinohara M, Mathieu D, et al. Novel integrative methodology for engineering large liver tissue equivalents based on three-dimensional scaffold fabrication and cellular aggregate assembly. *Biofabrication*. 2016;8(3):035016.

90. Lysy PA, Najimi M, Stéphenne X, Bourgois A, Smets F, Sokal EM. Liver cell transplantation for Crigler-Najjar syndrome type I: update and perspectives. *World J Gastroenterol.* 2008;14(22):3464-70.
91. Meyburg J, Das AM, Hoerster F, Lindner M, Kriegbaum H, Engelmann G, et al. One liver for four children: first clinical series of liver cell transplantation for severe neonatal urea cycle defects. *Transplantation.* 2009;87(5):636-41.
92. Dhawan A, Puppi J, Hughes RD, Mitry RR. Human hepatocyte transplantation: current experience and future challenges. *Nature Reviews Gastroenterology and Hepatology.* 2010;7(5):288-98.
93. Soltys KA, Soto-Gutiérrez A, Nagaya M, Baskin KM, Deutsch M, Ito R, et al. Barriers to the successful treatment of liver disease by hepatocyte transplantation. *Journal of hepatology.* 2010;53(4):769-74.
94. Badylak SF, Weiss DJ, Caplan A, Macchiarini P. Engineered whole organs and complex tissues. *The Lancet.* 2012;379(9819):943-52.
95. Uygun BE, Soto-Gutierrez A, Yagi H, Izamis M-L, Guzzardi MA, Shulman C, et al. Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix. *Nat Med.* 2010;16(7):814-20.
96. Barakat O, Abbasi S, Rodriguez G, Rios J, Wood RP, Ozaki C, et al. Use of decellularized porcine liver for engineering humanized liver organ. *J Surg Res.* 2012;173(1):e11-e25.
97. Visconti RP, Kasyanov V, Gentile C, Zhang J, Markwald RR, Mironov V. Towards organ printing: engineering an intra-organ branched vascular tree. *Expert opinion on biological therapy.* 2010;10(3):409-20.
98. Pérez-Pomares JM, Foty RA. Tissue fusion and cell sorting in embryonic development and disease: biomedical implications. *Bioessays.* 2006;28(8):809-21.
99. Lewis PL, Shah RN. 3D Printing for Liver Tissue Engineering: Current Approaches and Future Challenges. *Current Transplantation Reports.* 2016;3(1):100-8.
100. Guillotin B, Guillemot F. Cell patterning technologies for organotypic tissue fabrication. *Trends Biotechnol.* 2011;29(4):183-90.
101. Little T, Mironov V, Mehesz AN, Markwald R, Sugi Y, Lessner SM, et al. Engineering a 3D, biological construct: representative research in the South Carolina Project for Organ Biofabrication. *Biofabrication.* 2011;3(3):030202.
102. Khatiwala C, Law R, Shepherd B, Dorfman S, Csete M. 3D cell bioprinting for regenerative medicine research and therapies. *Gene Ther Regul.* 2012;7(01):1230004.
103. Fatehullah A, Tan SH, Barker N. Organoids as an in vitro model of human development and disease. *Nat Cell Biol.* 2016;18(3):246-54.
104. Takebe T, Sekine K, Enomura M, Koike H, Kimura M, Ogaeri T, et al. Vascularized and functional human liver from an iPSC-derived organ bud transplant. *Nature.* 2013;499(7459):481-4.
105. Michalopoulos GK. Liver regeneration: molecular mechanisms of growth control. *The FASEB Journal.* 1990;4(2):176-87.
106. Chen Y, Wong PP, Sjeklocha L, Steer CJ, Sahin MB. Mature hepatocytes exhibit unexpected plasticity by direct dedifferentiation into liver progenitor cells in culture. *Hepatology.* 2012;55(2):563-74.
107. Gomez-Lechon M, Donato M, Lahoz A, Castell J. Cell lines: a tool for in vitro drug metabolism studies. *Curr Drug Metab.* 2008;9(1):1-11.
108. Bader A, Rinkes IHB, Closs EI, Ryan CM, Toner M, Cunningham JM, et al. A stable long-term hepatocyte culture system for studies of physiologic processes: cytokine stimulation of the acute phase response in rat and human hepatocytes. *Biotechnol Prog.* 1992;8(3):219-25.
109. LeCluyse EL, Bullock PL, Parkinson A, Hochman JH. Cultured rat hepatocytes. *Models for assessing drug absorption and metabolism: Springer;* 1996. p. 121-59.
110. Castell JV, Jover R, Martinez-Jimenez CP, Gomez-Lechon MJ. Hepatocyte cell lines: their use, scope and limitations in drug metabolism studies. *Expert opinion on drug metabolism & toxicology.* 2006;2(2):183-212.

111. Li M, Yuan H, Li N, Song G, Zheng Y, Baratta M, et al. Identification of interspecies difference in efflux transporters of hepatocytes from dog, rat, monkey and human. *Eur J Pharm Sci.* 2008;35(1):114-26.
112. te Velde AA, Ladiges NC, Flendrig LM, Chamuleau RA. Functional activity of isolated pig hepatocytes attached to different extracellular matrix substrates. Implication for application of pig hepatocytes in a bioartificial liver. *Journal of hepatology.* 1995;23(2):184-92.
113. Pascher A, Sauer I, Neuhaus P. Analysis of allogeneic versus xenogeneic auxiliary organ perfusion in liver failure reveals superior efficacy of human livers. *The International journal of artificial organs.* 2002;25(10):1006-12.
114. Kelly JH, Darlington GJ. Modulation of the liver specific phenotype in the human hepatoblastoma line Hep G2. *In vitro cellular & developmental biology.* 1989;25(2):217-22.
115. Ellis AJ, Hughes RD, Wendon JA, Dunne J, Langley PG, Kelly JH, et al. Pilot-controlled trial of the extracorporeal liver assist device in acute liver failure. *Hepatology.* 1996;24(6):1446-51.
116. Liu J, Pan J, Naik S, Santangini H, Trenkler D, Thompson N, et al. Characterization and evaluation of detoxification functions of a nontumorigenic immortalized porcine hepatocyte cell line (HepLiu). *Cell transplantation.* 1998;8(3):219-32.
117. Yoon J, Lee H, Lee J, Park J, Kim C. Development of a non-transformed human liver cell line with differentiated-hepatocyte and urea-synthetic functions: applicable for bioartificial liver. *The International journal of artificial organs.* 1999;22(11):769-77.
118. Gripon P, Rumin S, Urban S, Le Seyec J, Glaise D, Cannie I, et al. Infection of a human hepatoma cell line by hepatitis B virus. *Proceedings of the National Academy of Sciences.* 2002;99(24):15655-60.
119. Allen JW, Bhatia SN. Engineering liver therapies for the future. *Tissue Eng.* 2002;8(5):725-37.
120. Bhatia SN, Underhill GH, Zaret KS, Fox JJ. Cell and tissue engineering for liver disease. *Science translational medicine.* 2014;6(245):245sr2-sr2.
121. Shan J, Schwartz RE, Ross NT, Logan DJ, Thomas D, Duncan SA, et al. Identification of small molecules for human hepatocyte expansion and iPS differentiation. *Nat Chem Biol.* 2013;9(8):514-20.
122. Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proceedings of the National Academy of Sciences.* 1981;78(12):7634-8.
123. Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. *Nature.* 1981;292(5819):154-6.
124. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, et al. Embryonic stem cell lines derived from human blastocysts. *science.* 1998;282(5391):1145-7.
125. Pera MF, Trounson AO. Human embryonic stem cells: prospects for development. *Development.* 2004;131(22):5515-25.
126. Jensen J, Hyllner J, Björquist P. Human embryonic stem cell technologies and drug discovery. *J Cell Physiol.* 2009;219(3):513-9.
127. Yildirimman R, Brolén G, Vilardell M, Eriksson G, Synnergren J, Gmuender H, et al. Human embryonic stem cell derived hepatocyte-like cells as a tool for in vitro hazard assessment of chemical carcinogenicity. *Toxicol Sci.* 2011:kfr225.
128. Greenhough S, Medine CN, Hay DC. Pluripotent stem cell derived hepatocyte like cells and their potential in toxicity screening. *Toxicology.* 2010;278(3):250-5.
129. Hay DC, Fletcher J, Payne C, Terrace JD, Gallagher RC, Snoeys J, et al. Highly efficient differentiation of hESCs to functional hepatic endoderm requires ActivinA and Wnt3a signaling. *Proceedings of the National Academy of Sciences.* 2008;105(34):12301-6.
130. Touboul T, Hannan NR, Corbineau S, Martinez A, Martinet C, Branchereau S, et al. Generation of functional hepatocytes from human embryonic stem cells under chemically defined conditions that recapitulate liver development. *Hepatology.* 2010;51(5):1754-65.

131. Duan Y, Ma X, Zou W, Wang C, Bahbahan IS, Ahuja TP, et al. Differentiation and characterization of metabolically functioning hepatocytes from human embryonic stem cells. *Stem Cells*. 2010;28(4):674-86.
132. Asplund A, Pradip A, van Giezen M, Aspegren A, Choukair H, Rehnström M, et al. One standardized differentiation procedure robustly generates homogenous hepatocyte cultures displaying metabolic diversity from a large panel of human pluripotent stem cells. *Stem Cell Reviews and Reports*. 2016;12(1):90-104.
133. Si-Tayeb K, Noto FK, Nagaoka M, Li J, Battle MA, Duris C, et al. Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. *Hepatology*. 2010;51(1):297-305.
134. Schwartz R, Fleming H, Khetani S, Bhatia S. Pluripotent stem cell-derived hepatocyte-like cells. *Biotechnol Adv*. 2014;32(2):504-13.
135. Fuchs E, Tumber T, Guasch G. Socializing with the neighbors: stem cells and their niche. *Cell*. 2004;116(6):769-78.
136. Wilson JW, Leduc EH. Role of cholangioles in restoration of the liver of the mouse after dietary injury. *The Journal of pathology and bacteriology*. 1958;76(2):441-9.
137. Herrera MB, Bruno S, Buttiglieri S, Tetta C, Gatti S, Deregibus MC, et al. Isolation and characterization of a stem cell population from adult human liver. *Stem Cells*. 2006;24(12):2840-50.
138. Chen Q, Liu F, Zhao G, Xue L, Hu R, Wu H, et al. In-vitro amplification of oval cells with preservation of stem cell phenotype. *Zhonghua bing li xue za zhi Chinese journal of pathology*. 2010;39(8):548-52.
139. Carraro A, Flaibani M, Cillo U, Michelotto L, Magrofuoco E, Buggio M, et al. A combining method to enhance the in vitro differentiation of hepatic precursor cells. *Tissue Engineering Part C: Methods*. 2010;16(6):1543-51.
140. Zhou W-L, Medine CN, Zhu L, Hay DC. Stem cell differentiation and human liver disease. *World J Gastroenterol*. 2012;18(17):2018-25.
141. Schmelzer E, Zhang L, Bruce A, Wauthier E, Ludlow J, Yao H-I, et al. Human hepatic stem cells from fetal and postnatal donors. *J Exp Med*. 2007;204(8):1973-87.
142. Lee J-H, Park H-J, Kim Y-A, Lee D-H, Noh J-K, Kwon C, et al., editors. The phenotypic characteristic of liver-derived stem cells from adult human deceased donor liver. *Transplantation proceedings*; 2012: Elsevier.
143. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*. 2006;126(4):663-76.
144. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*. 2007;131(5):861-72.
145. Yamanaka S. A fresh look at iPS cells. *Cell*. 2009;137(1):13-7.
146. Takayama K, Inamura M, Kawabata K, Katayama K, Higuchi M, Tashiro K, et al. Efficient generation of functional hepatocytes from human embryonic stem cells and induced pluripotent stem cells by HNF4 α transduction. *Mol Ther*. 2012;20(1):127-37.
147. Yu Y, Liu H, Ikeda Y, Amiot BP, Rinaldo P, Duncan SA, et al. Hepatocyte-like cells differentiated from human induced pluripotent stem cells: Relevance to cellular therapies. *Stem cell research*. 2012;9(3):196-207.
148. Lee SB, Seo D, Choi D, Park KY, Holczbauer A, Marquardt JU, et al. Contribution of Hepatic Lineage Stage-Specific Donor Memory to the Differential Potential of Induced Mouse Pluripotent Stem Cells. *Stem Cells*. 2012;30(5):997-1007.
149. Cho CH, Park J, Nagrath D, Tilles AW, Berthiaume F, Toner M, et al. Oxygen uptake rates and liver-specific functions of hepatocyte and 3T3 fibroblast co-cultures. *Biotechnol Bioeng*. 2007;97(1):188-99.
150. Allen JW, Bhatia SN. Formation of steady-state oxygen gradients in vitro: Application to liver zonation. *Biotechnol Bioeng*. 2003;82(3):253-62.
151. Broughan TA, Naukam R, Tan C, Van De Wiele CJ, Refai H, Teague TK. Effects of hepatic zonal oxygen levels on hepatocyte stress responses. *J Surg Res*. 2008;145(1):150-60.

152. Wang Y, Susando T, Lei X, Anene-Nzelu C, Zhou H, Liang LH, et al. Current development of bioreactors for extracorporeal bioartificial liver (Review). *Biointerphases*. 2010;5(3):FA116-FA31.
153. Demetriou AA, Brown Jr RS, Busuttill RW, Fair J, McGuire BM, Rosenthal P, et al. Prospective, randomized, multicenter, controlled trial of a bioartificial liver in treating acute liver failure. *Annals of surgery*. 2004;239(5):660-70.
154. Sussman NL, Gislason GT, Conlin CA, Kelly JH. The Hepatix extracorporeal liver assist device: initial clinical experience. *Artif Organs*. 1994;18(5):390-6.
155. Flendrig LM, la Soe JW, Jörning GG, Steenbeek A, Karlsen OT, Bovée WM, et al. In vitro evaluation of a novel bioreactor based on an integral oxygenator and a spirally wound nonwoven polyester matrix for hepatocyte culture as small aggregates. *Journal of hepatology*. 1997;26(6):1379-92.
156. Patzer JF, Mazariegos GV, Lopez R, Molmenti E, Gerber D, Riddervold F, et al. Novel bioartificial liver support system: preclinical evaluation. *Ann N Y Acad Sci*. 1999;875(1):340-52.
157. Mazariegos GV, Patzer JF, Lopez RC, Giraldo M, DeVera ME, Grogan TA, et al. First clinical use of a novel bioartificial liver support system (BLSS). *Am J Transplantation*. 2002;2(3):260-6.
158. Sauer I, Kardassis D, Zeillinger K, Pascher A, Gruenwald A, Pless G, et al. Clinical extracorporeal hybrid liver support—phase I study with primary porcine liver cells. *Xenotransplantation*. 2003;10(5):460-9.
159. Morsiani E, Brogli M, Galavotti D, Bellini T, Ricci D, Pazzi P, et al. Long-Term Expression of Highly Differentiated Functions by Isolated Porcine Hepatocytes Perfused in a Radial-Flow Bioreactor. *Artif Organs*. 2001;25(9):740-8.
160. De Bartolo L, Salerno S, Morelli S, Giorno L, Rende M, Memoli B, et al. Long-term maintenance of human hepatocytes in oxygen-permeable membrane bioreactor. *Biomaterials*. 2006;27(27):4794-803.
161. De Bartolo L, Salerno S, Curcio E, Piscioneri A, Rende M, Morelli S, et al. Human hepatocyte functions in a crossed hollow fiber membrane bioreactor. *Biomaterials*. 2009;30(13):2531-43.
162. Curcio E, Salerno S, Barbieri G, De Bartolo L, Drioli E, Bader A. Mass transfer and metabolic reactions in hepatocyte spheroids cultured in rotating wall gas-permeable membrane system. *Biomaterials*. 2007;28(36):5487-97.
163. Hu W-S, Friend JR, Wu FJ, Sielaff T, Peshwa MV, Lazar A, et al. Development of a bioartificial liver employing xenogeneic hepatocytes. *Cytotechnology*. 1997;23(1-3):29-38.
164. Nagaki M, Miki K, Kim Y-I, Ishiyama H, Hirahara I, Takahashi H, et al. Development and characterization of a hybrid bioartificial liver using primary hepatocytes entrapped in a basement membrane matrix. *Dig Dis Sci*. 2001;46(5):1046-56.
165. Jasmund I, Langsch A, Simmoteit R, Bader A. Cultivation of Primary Porcine Hepatocytes in an OXY-HFB for Use as a Bioartificial Liver Device. *Biotechnol Prog*. 2002;18(4):839-46.
166. Lu H-F, Lim WS, Zhang P-C, Chia SM, Yu H, Mao H-Q, et al. Galactosylated poly (vinylidene difluoride) hollow fiber bioreactor for hepatocyte culture. *Tissue Eng*. 2005;11(11-12):1667-77.
167. Memoli B, De Bartolo L, Favia P, Morelli S, Lopez LC, Procino A, et al. Fetuin-A gene expression, synthesis and release in primary human hepatocytes cultured in a galactosylated membrane bioreactor. *Biomaterials*. 2007;28(32):4836-44.
168. Chao P, Maguire T, Novik E, Cheng K-C, Yarmush M. Evaluation of a microfluidic based cell culture platform with primary human hepatocytes for the prediction of hepatic clearance in human. *Biochem Pharmacol*. 2009;78(6):625-32.
169. Novik E, Maguire TJ, Chao P, Cheng K, Yarmush ML. A microfluidic hepatic coculture platform for cell-based drug metabolism studies. *Biochem Pharmacol*. 2010;79(7):1036-44.
170. Soldatow VY, LeCluyse EL, Griffith LG, Rusyn I. In vitro models for liver toxicity testing. *Toxicology research*. 2013;2(1):23-39.

171. Park J, Li Y, Berthiaume F, Toner M, Yarmush ML, Tilles AW. Radial flow hepatocyte bioreactor using stacked microfabricated grooved substrates. *Biotechnol Bioeng.* 2008;99(2):455-67.
172. Sinz M, Wallace G, Sahi J. Current industrial practices in assessing CYP450 enzyme induction: preclinical and clinical. *The AAPS journal.* 2008;10(2):391-400.
173. Coecke S, Rogiers V, Bayliss M, Castell J, Doehmer J, Fabre G, et al. The use of long-term hepatocyte cultures for detecting induction of drug metabolising enzymes: the current status. *ATLA-Nottingham.* 1999;27:579-638.
174. Nakamura T, Nakayama Y, Ichihara A. Reciprocal modulation of growth and liver functions of mature rat hepatocytes in primary culture by an extract of hepatic plasma membranes. *J Biol Chem.* 1984;259(13):8056-8.
175. Zeigerer A, Wuttke A, Marsico G, Seifert S, Kalaidzidis Y, Zerial M. Functional properties of hepatocytes in vitro are correlated with cell polarity maintenance. *Exp Cell Res.* 2017;350(1):242-52.
176. Miranda JP, Rodrigues A, Tostoes RM, Leite S, Zimmerman H, Carrondo MJ, et al. Extending hepatocyte functionality for drug-testing applications using high-viscosity alginate-encapsulated three-dimensional cultures in bioreactors. *Tissue Engineering Part C: Methods.* 2010;16(6):1223-32.
177. Abu-Absi SF, Friend JR, Hansen LK, Hu W-S. Structural polarity and functional bile canaliculi in rat hepatocyte spheroids. *Exp Cell Res.* 2002;274(1):56-67.
178. Miranda JP, Leite SB, Muller-Vieira U, Rodrigues A, Carrondo MJ, Alves PM. Towards an extended functional hepatocyte in vitro culture. *Tissue Engineering Part C: Methods.* 2008;15(2):157-67.
179. Sakai Y, Yamagami S, Nakazawa K. Comparative analysis of gene expression in rat liver tissue and monolayer-and spheroid-cultured hepatocytes. *Cells Tissues Organs.* 2009;191(4):281-8.
180. Brophy CM, Luebke-Wheeler JL, Amiot BP, Khan H, Rimmel RP, Rinaldo P, et al. Rat hepatocyte spheroids formed by rocked technique maintain differentiated hepatocyte gene expression and function. *Hepatology.* 2009;49(2):578-86.
181. Gunness P, Mueller D, Shevchenko V, Heinzele E, Ingelman-Sundberg M, Noor F. 3D organotypic cultures of human HepaRG cells: a tool for in vitro toxicity studies. *Toxicol Sci.* 2013;133(1):67-78.
182. Sarvi F, Arbatan T, Chan PPY, Shen W. A novel technique for the formation of embryoid bodies inside liquid marbles. *Rsc Advances.* 2013;3(34):14501-8.
183. Nakazawa K, Izumi Y, Fukuda J, Yasuda T. Hepatocyte spheroid culture on a polydimethylsiloxane chip having microcavities. *Journal of Biomaterials Science, Polymer Edition.* 2006;17(8):859-73.
184. Hardelauf H, Frimat J-P, Stewart JD, Schormann W, Chiang Y-Y, Lampen P, et al. Microarrays for the scalable production of metabolically relevant tumour spheroids: a tool for modulating chemosensitivity traits. *Lab on a Chip.* 2011;11(3):419-28.
185. Yang M-J, Chen C-H, Lin P-J, Huang C-H, Chen W, Sung H-W. Novel method of forming human embryoid bodies in a polystyrene dish surface-coated with a temperature-responsive methylcellulose hydrogel. *Biomacromolecules.* 2007;8(9):2746-52.
186. Okudaira T, Amimono N, Mizumoto H, Kajiwarra T. Formation of three-dimensional hepatic tissue by the bottom-up method using spheroids. *J Biosci Bioeng.* 2016;122(2):213-8.
187. Nakazawa K, Ijima H, Fukuda J, Sakiyama R, Yamashita Y, Shimada M, et al. Development of a hybrid artificial liver using polyurethane foam/hepatocyte spheroid culture in a preclinical pig experiment. *The International journal of artificial organs.* 2002;25(1):51-60.
188. Du Y, Chia S-m, Han R, Chang S, Tang H, Yu H. 3D hepatocyte monolayer on hybrid RGD/galactose substratum. *Biomaterials.* 2006;27(33):5669-80.
189. Chua K-N, Lim W-S, Zhang P, Lu H, Wen J, Ramakrishna S, et al. Stable immobilization of rat hepatocyte spheroids on galactosylated nanofiber scaffold. *Biomaterials.* 2005;26(15):2537-47.

190. Wells RG. The role of matrix stiffness in regulating cell behavior. *Hepatology*. 2008;47(4):1394-400.
191. Mattei G, Giusti S, Ahluwalia A. Design criteria for generating physiologically relevant in vitro models in bioreactors. *Processes*. 2014;2(3):548-69.
192. Tong WH, Fang Y, Yan J, Hong X, Singh NH, Wang SR, et al. Constrained spheroids for prolonged hepatocyte culture. *Biomaterials*. 2016;80:106-20.
193. Carraro A, Hsu W-M, Kulig KM, Cheung WS, Miller ML, Weinberg EJ, et al. In vitro analysis of a hepatic device with intrinsic microvascular-based channels. *Biomed Microdevices*. 2008;10(6):795-805.
194. Korin N, Bransky A, Khoury M, Dinnar U, Levenberg S. Design of well and groove microchannel bioreactors for cell culture. *Biotechnol Bioeng*. 2009;102(4):1222-30.
195. Zhang MY, Lee PJ, Hung PJ, Johnson T, Lee LP, Mofrad MR. Microfluidic environment for high density hepatocyte culture. *Biomed Microdevices*. 2008;10(1):117-21.
196. Tostoes RM, Leite SB, Miranda JP, Sousa M, Wang DI, Carrondo MJ, et al. Perfusion of 3D encapsulated hepatocytes—A synergistic effect enhancing long-term functionality in bioreactors. *Biotechnol Bioeng*. 2011;108(1):41-9.
197. Khetani SR, Bhatia SN. Microscale culture of human liver cells for drug development. *Nat Biotechnol*. 2008;26(1):120-6.
198. Domansky K, Inman W, Serdy J, Dash A, Lim MH, Griffith LG. Perfused multiwell plate for 3D liver tissue engineering. *Lab on a chip*. 2010;10(1):51-8.
199. Tostoes RM, Leite SB, Serra M, Jensen J, Björquist P, Carrondo MJ, et al. Human liver cell spheroids in extended perfusion bioreactor culture for repeated-dose drug testing. *Hepatology*. 2012;55(4):1227-36.
200. Kocarek TA, Schuetz EG, Strom SC, Fisher RA, Guzelian PS. Comparative analysis of cytochrome P4503A induction in primary cultures of rat, rabbit, and human hepatocytes. *Drug Metab Disposition*. 1995;23(3):415-21.
201. Weigand K, Otto I. Secretion of serum albumin by enzymatically isolated rat liver cells. *FEBS Lett*. 1974;46(1):127-9.
202. Watts P, Smith MD, Edwards I, Zammit V, Brown V, Grant H. The influence of medium composition on the maintenance of cytochrome P-450, glutathione content and urea synthesis: a comparison of rat and sheep primary hepatocyte cultures. *Journal of hepatology*. 1995;23(5):605-12.
203. Yamaoka M, Mirata K, Ogata I, Tomiya T, Nagoshi S, Mochida S, et al. Enhancement of albumin production by hepatocyte growth factor in rat hepatocytes: distinction in mode of action from stimulation of DNA synthesis. *Liver*. 1998;18(1):52-9.
204. Pritchard JF, Jurima-Romet M, Reimer ML, Mortimer E, Rolfe B, Cayen MN. Making better drugs: Decision gates in non-clinical drug development. *Nature Reviews Drug Discovery*. 2003;2(7):542-53.
205. Gómez-Lechón MJ, Castell JV, Donato MT. An update on metabolism studies using human hepatocytes in primary culture. 2008.
206. Arterburn LM, Zurlo J, Yager JD, Overton RM, Heifetz AH. A morphological study of differentiated hepatocytes in vitro. *Hepatology*. 1995;22(1):175-87.
207. Bale SS, Verneti L, Senutovitch N, Jindal R, Hegde M, Gough A, et al. In vitro platforms for evaluating liver toxicity. *Exp Biol Med*. 2014;239(9):1180-91.
208. LeCluyse EL, Witek RP, Andersen ME, Powers MJ. Organotypic liver culture models: meeting current challenges in toxicity testing. *Crit Rev Toxicol*. 2012;42(6):501-48.
209. Kmiec Z. Cooperation of liver cells in health and disease: Springer Science & Business Media; 2001.
210. Shah V, Haddad FG, Garcia-Cardena G, Frangos JA, Mennone A, Groszmann RJ, et al. Liver sinusoidal endothelial cells are responsible for nitric oxide modulation of resistance in the hepatic sinusoids. *J Clin Invest*. 1997;100(11):2923.
211. DeLeve LD, Wang X, Guo Y. Sinusoidal endothelial cells prevent rat stellate cell activation and promote reversion to quiescence. *Hepatology*. 2008;48(3):920-30.

212. Steinberg P, Lafranconi WM, Wolf CR, Waxman DJ, Oesch F, Friedberg T. Xenobiotic metabolizing enzymes are not restricted to parenchymal cells in rat liver. *Mol Pharmacol*. 1987;32(4):463-70.
213. DeLeve LD. Dacarbazine toxicity in murine liver cells: a model of hepatic endothelial injury and glutathione defense. *J Pharmacol Exp Ther*. 1994;268(3):1261-70.
214. Weiner FR, Giambrone MA, Czaja MJ, Shah A, Annoni G, Takahashi S, et al. Ito-cell gene expression and collagen regulation. *Hepatology*. 1990;11(1):111-7.
215. Wang Y, Yao HL, Cui CB, Wauthier E, Barbier C, Costello MJ, et al. Paracrine signals from mesenchymal cell populations govern the expansion and differentiation of human hepatic stem cells to adult liver fates. *Hepatology*. 2010;52(4):1443-54.
216. Bader A, Knop E, Kern A, Böker K, Frühauf N, Crome O, et al. 3-D coculture of hepatic sinusoidal cells with primary hepatocytes—design of an organotypical model. *Exp Cell Res*. 1996;226(1):223-33.
217. Riccalton-Banks L, Liew C, Bhandari R, Fry J, Shakesheff K. Long-term culture of functional liver tissue: three-dimensional coculture of primary hepatocytes and stellate cells. *Tissue Eng*. 2003;9(3):401-10.
218. Krause P, Saghatolislam F, Koenig S, Unthan-Fechner K, Probst I. Maintaining hepatocyte differentiation in vitro through co-culture with hepatic stellate cells. *In Vitro Cellular & Developmental Biology-Animal*. 2009;45(5-6):205-12.
219. Zeilinger K, Holland G, Sauer IM, Efimova E, Kardassis D, Obermayer N, et al. Time course of primary liver cell reorganization in three-dimensional high-density bioreactors for extracorporeal liver support: an immunohistochemical and ultrastructural study. *Tissue Eng*. 2004;10(7-8):1113-24.
220. Pless G, Steffen I, Zeilinger K, Sauer IM, Katenz E, Kehr DC, et al. Evaluation of primary human liver cells in bioreactor cultures for extracorporeal liver support on the basis of urea production. *Artif Organs*. 2006;30(9):686-94.
221. Messina A, Morelli S, Forgacs G, Barbieri G, Drioli E, De Bartolo L. Self-assembly of tissue spheroids on polymeric membranes. *Journal of tissue engineering and regenerative medicine*. 2015.
222. Morelli S, Piscioneri A, Messina A, Salerno S, Al-Fageeh MB, Drioli E, et al. Neuronal growth and differentiation on biodegradable membranes. *Journal of tissue engineering and regenerative medicine*. 2015;9(2):106-17.
223. Kasuya J, Sudo R, Masuda G, Mitaka T, Ikeda M, Tanishita K. Reconstruction of hepatic stellate cell-incorporated liver capillary structures in small hepatocyte tri-culture using microporous membranes. *Journal of tissue engineering and regenerative medicine*. 2015;9(3):247-56.
224. Xiao W, Perry G, Komori K, Sakai Y. New physiologically-relevant liver tissue model based on hierarchically cocultured primary rat hepatocytes with liver endothelial cells. *Integrative Biology*. 2015;7(11):1412-22.
225. Kostadinova R, Boess F, Applegate D, Suter L, Weiser T, Singer T, et al. A long-term three dimensional liver co-culture system for improved prediction of clinically relevant drug-induced hepatotoxicity. *Toxicol Appl Pharmacol*. 2013;268(1):1-16.
226. Prodanov L, Jindal R, Bale SS, Hegde M, McCarty WJ, Golberg I, et al. Long-term maintenance of a microfluidic 3D human liver sinusoid. *Biotechnol Bioeng*. 2016;113(1):241-6.
227. Thomas RJ, Bhandari R, Barrett DA, Bennett AJ, Fry JR, Powe D, et al. The effect of three-dimensional co-culture of hepatocytes and hepatic stellate cells on key hepatocyte functions in vitro. *Cells Tissues Organs*. 2005;181(2):67-79.
228. Ramadori G, Moriconi F, Malik I, Dudas J. Physiology and pathophysiology of liver inflammation, damage and repair. *J Physiol pharmacol*. 2008;59(Suppl 1):107-17.
229. Salerno S, Morelli S, Drioli E, Bader A, de Bartolo L. Biofabrication of Layered Membrane Systems by Using Human Hepatocytes and Endothelial Cells: A Comparative Study. *Current Tissue Engineering*. 2013;2(2):109-18.

230. Bhatia S, Balis U, Yarmush M, Toner M. Effect of cell–cell interactions in preservation of cellular phenotype: cocultivation of hepatocytes and nonparenchymal cells. *The FASEB Journal*. 1999;13(14):1883-900.
231. Curcio E, Piscioneri A, Salerno S, Tasselli F, Morelli S, Drioli E, et al. Human lymphocytes cultured in 3-D bioreactors: Influence of configuration on metabolite transport and reactions. *Biomaterials*. 2012;33(33):8296-303.
232. Jung F, Richardson TH, Raucy JL, Johnson EF. Diazepam Metabolism by cDNA-Expressed Human 2C P450s Identification of P4502C18 and P4502C19 as LowKM Diazepam N-Demethylases. *Drug Metab Disposition*. 1997;25(2):133-9.
233. Onof S, Hatanaka T, Miyazawa S, Tsutsui M, Aoyama T, Gonzalez F, et al. Human liver microsomal diazepam metabolism using cDNA-expressed cytochrome P450s: role of CYP2B6, 2C19 and the 3A subfamily. *Xenobiotica*. 1996;26(11):1155-66.
234. Xiao W, Shinohara M, Komori K, Sakai Y, Matsui H, Osada T. The importance of physiological oxygen concentrations in the sandwich cultures of rat hepatocytes on gas-permeable membranes. *Biotechnol Prog*. 2014;30(6):1401-10.
235. Gerlach JC, Zeilinger K, Patzer I, JF. Bioartificial liver systems: why, what, whither? 2008.
236. Van de Kerkhove M, Di Florio E, Scuderi V, Mancini A, Belli A, Bracco A, et al. Phase I clinical trial with the AMC-bioartificial liver. *International journal of artificial organs*. 2002;25(10):950-9.
237. van de Kerkhove MP, Chamuleau R, Van Gulik T. Clinical application of bioartificial liver support systems. *Encephalopathy and nitrogen metabolism in liver failure*: Springer; 2003. p. 389-406.
238. Streetz KL. Bio-artificial liver devices—tentative, but promising progress. *Journal of hepatology*. 2008;48(2):189-91.
239. Baquerizo A, Mhoyan A, Kearns-Jonker M, Arnaout WS, Shackleton C, Busuttill RW, et al. Characterization of human xenoreactive antibodies in liver failure patients exposed to pig hepatocytes after bioartificial liver treatment: An Ex Vivo Model of Pig to Human Xenotransplantation1, 2. *Transplantation*. 1999;67(1):5-18.
240. Sussman NL, Kelly JH. Improved liver function following treatment with an extracorporeal liver assist device. *Artif Organs*. 1993;17(1):27-30.
241. Zeindl-Eberhart E, Rabes HM. Variant protein patterns in hepatomas and transformed liver cell lines as determined by high resolution two-dimensional gel electrophoresis (2DE). *Carcinogenesis*. 1992;13(7):1177-83.
242. Alison MR, Poulson R, Jeffery R, Dhillon AP, Quaglia A, Jacob J, et al. Cell differentiation: hepatocytes from non-hepatic adult stem cells. *Nature*. 2000;406:257.
243. Fiegel HC, Lioznov MV, Cortes-Dericks L, Lange C, Kluth D, Fehse B, et al. Liver-Specific Gene Expression in Cultured Human Hematopoietic Stem Cells. *Stem Cells*. 2003;21(1):98-104.
244. Hong SH, Gang EJ, Jeong JA, Ahn C, Hwang SH, Yang IH, et al. In vitro differentiation of human umbilical cord blood-derived mesenchymal stem cells into hepatocyte-like cells. *Biochem Biophys Res Commun*. 2005;330(4):1153-61.
245. Banas A, Teratani T, Yamamoto Y, Tokuhara M, Takeshita F, Quinn G, et al. Adipose tissue-derived mesenchymal stem cells as a source of human hepatocytes. *Hepatology*. 2007;46(1):219-28.
246. Lee KD, Kuo TKC, Whang-Peng J, Chung YF, Lin CT, Chou SH, et al. In vitro hepatic differentiation of human mesenchymal stem cells. *Hepatology*. 2004;40(6):1275-84.
247. Schmelzer E, Triolo F, Turner ME, Thompson RL, Zeilinger K, Reid LM, et al. Three-dimensional perfusion bioreactor culture supports differentiation of human fetal liver cells. *Tissue Engineering Part A*. 2010;16(6):2007-16.
248. Lee J-H, Park H-J, Jang I, Kim H-E, Lee D-H, Park J-K, et al., editors. In vitro differentiation of human liver-derived stem cells with mesenchymal characteristics into immature hepatocyte-like cells. *Transplantation proceedings*; 2014: Elsevier.
249. Michalopoulos GK. Liver regeneration after partial hepatectomy: critical analysis of mechanistic dilemmas. *The American journal of pathology*. 2010;176(1):2-13.

250. Fausto N. Liver regeneration and repair: hepatocytes, progenitor cells, and stem cells. *Hepatology*. 2004;39(6):1477-87.
251. Tamama K, Fan VH, Griffith LG, Blair HC, Wells A. Epidermal growth factor as a candidate for ex vivo expansion of bone marrow-derived mesenchymal stem cells. *Stem Cells*. 2006;24(3):686-95.
252. Salehinejad P, Alitheen NB, Mandegary A, Nematollahi-mahani SN, Janzamin E. Effect of EGF and FGF on the expansion properties of human umbilical cord mesenchymal cells. *In Vitro Cellular & Developmental Biology-Animal*. 2013;49(7):515-23.
253. Jung J, Zheng M, Goldfarb M, Zaret KS. Initiation of mammalian liver development from endoderm by fibroblast growth factors. *Science*. 1999;284(5422):1998-2003.
254. Ghaedi M, Tuleuova N, Zern MA, Wu J, Revzin A. Bottom-up signaling from HGF-containing surfaces promotes hepatic differentiation of mesenchymal stem cells. *Biochem Biophys Res Commun*. 2011;407(2):295-300.
255. Forte G, Minieri M, Cossa P, Antenucci D, Sala M, Gnocchi V, et al. Hepatocyte growth factor effects on mesenchymal stem cells: proliferation, migration, and differentiation. *Stem Cells*. 2006;24(1):23-33.
256. Chivu M, Dima SO, Stancu CI, Dobra C, Usatescu V, Necula LG, et al. In vitro hepatic differentiation of human bone marrow mesenchymal stem cells under differential exposure to liver-specific factors. *Translational Research*. 2009;154(3):122-32.
257. Zhou QJ, Xiang LX, Shao JZ, Hu RZ, Lu YL, Yao H, et al. In vitro differentiation of hepatic progenitor cells from mouse embryonic stem cells induced by sodium butyrate. *J Cell Biochem*. 2007;100(1):29-42.
258. Miyajima A, Kinoshita T, Tanaka M, Kamiya A, Mukoyama Y, Hara T. Role of Oncostatin M in hematopoiesis and liver development. *Cytokine Growth Factor Rev*. 2000;11(3):177-83.
259. Michalopoulos GK, Bowen WC, Mulé K, Luo J. HGF-, EGF-, and dexamethasone-induced gene expression patterns during formation of tissue in hepatic organoid cultures. *Gene Expression*. 2003;11(2):55-75.
260. Seo MJ, Suh SY, Bae YC, Jung JS. Differentiation of human adipose stromal cells into hepatic lineage in vitro and in vivo. *Biochem Biophys Res Commun*. 2005;328(1):258-64.
261. Pietrangelo A, Panduro A, Chowdhury JR, Shafritz DA. Albumin gene expression is down-regulated by albumin or macromolecule infusion in the rat. *J Clin Invest*. 1992;89(6):1755.