OVERCOMING MASS TRANSFER BARRIERS IN SANDWICH CONFIGURATION FOR PRIMARY HEPATOCYTES CULTURE

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TABLE OF CONTENTS

SUMMARY

This thesis explored two novel ways to encounter the inherent mass transfer barriers of conventional sandwich configuration for primary hepatocytes culture combining principles and technologies from tissue engineering, chemistry and bioreactor engineering.

Sandwiching hepatocytes between two layers of extra-cellular matrix support creates an intra-sandwich environment which differs from the extra-sandwich environment defined by culture medium. When the intra-sandwich environment was characterized, an albumin accumulation intra-sandwich environment in a conventional static hepatocytes sandwich culture was identified. This indicated that the mass transfer in the conventional sandwich configuration is limited. Further studies explored the effect of the mass transfer limitation to hepatocytes' functions in sandwich culture. Albumin accumulation in the intra-sandwich environment resulted in reduced hepatocytes functions in static culture.

To increase the mass transfer efficacy (indicated by effectively removal of albumin out of intra-sandwich environment), hepatocytes were cultured in a perfusion sandwich configuration by flowing culture medium at different flow rates above the upper extra-cellular matrix support on porous membrane in a flat plate sandwich perfusion culture bioreactor. It was found that albumin removal from the intra-sandwich environment cannot be effectively achieved by varying the perfusion rates without adversely affecting the hepatocytes functions. Based on the observation, we have designed a novel bioreactor with a separate drainage channel directly connected to the intra-sandwich environment, facilitating the removal of the metabolites and

supply of nutrients directly. The mass transfer efficacy can be effectively regulated by varying the drainage rates via the drainage channel without changing the perfusion rates, as indicated by the phenomena that intra-sandwich albumin level was effectively regulated by direct control of the drainage rates. Using the separate drainage system, an optimal level of the drainage rates and mass transfer efficacy can be maintained, which improved hepatocytes functions over the no-drainage controls.

Apart from the using of flow environment to improve mass transfer efficacy, we also focused on the conventional sandwich configuration itself and tried to improve the mass transfer efficacy by replacing the natural ECMs such as collagen, the main cause of mass transfer limitation, with the synthetic polymers with controllable physical and chemical properties. After trying with various functional polymers, an ideal synthetic sandwich configuration was identified by overlaying a novel 3D monolayer developed on galactosylated PET film with RGD conjugated polyethylene terephthalate (RGD-PET) membranes, which also possessed better mass transfer properties over ECM such as collagen. We proved that this configuration had the similar polarity genesis process as conventional sandwich configurations: reorganization of F-actin in cell-cell contact regions after 12h of sandwich culture; localization of bile canaliculi transporter (MRP2) into bile channel after 24h of sandwich culture; regaining of active bile secretion ability during the first several days of sandwich culture. Moreover, enhanced cell-cell interaction and improved hepatocytes functions over 14 days of culture were observed in the synthetic sandwich configuration, most likely due to the high mass transfer efficacy of this system.

LIST OF FIGURES AND TABLES

LIST OF SYMBOLS

ECM	Extra-cellular matrix
PET	Polyethylene terephthalate
RGD	Arg-Gly-Asp
MRP2	Multidrug resistance protein 2
BSA	Bovine serum albumin
FITC	Fluorescein 5'-isothiocyanate
PBS	Phosphate buffered saline
FDA	Fluorescein diacetate
XPS	X-ray Photoelectron Spectroscopy

Chapter 1 Introduction

1.1 Liver tissue engineering

1.1.1 Overview of tissue engineering

The field of tissue engineering, by integrating principles of engineering and life sciences, exploits living cells in a variety of ways to restore, maintain, or enhance tissues and organs [1]. Generally, the application of tissue engineering can be divided as therapeutic application, in which the tissue is either grown in a patient or outside the patient [2,3] and diagnostic applications, in which the tissue and culture models are engineered *in vitro* and used for testing drug metabolism, uptake, toxicity and, pathogenicity, etc [4-6].

In both applications, cultured cells need to be coaxed to grow on bioactive degradable matrix under properly engineered environment that provide the physical and chemical cues to induce the regeneration functions needed, such as guiding cells' differentiation ability and assembly process into three-dimensional (3D) tissues [7]. Current progress in tissue engineering is mainly limited in this step; those challenges include finding reliable sources of compatible cells [8-10], engineering of proper cell culture matrix (Biomaterials) [11-14], and the creating of novel bioreactors [15-18], which mimic the environment of the body and that are amenable to scale-up. With fast development of these areas recently, it is possible that laboratory-grown tissue replacements and cell models will become a common medical therapy during the early decades of the 21st century. However, we need to be aware of the problems such like whether tissue

engineers can preserve the product so that it has a long shelf-life? Is it possible to permit the fine control of tissue architecture for the engineered tissues to become clinically useful without tissue rejection? All of these questions are pending solving.

1.1.2 Applications of liver tissue engineering

Liver, the largest organ in the body, serves vital roles in the body's metabolization and detoxification function, while liver diseases present a large portion of healthcare problem worldwide with high incidents of cirrhosis, liver cancer and liver failure [19,20]. Although dramatic advances in surgical techniques and immuno-suppression have permitted the use of liver transplantation in the management of liver disease, the patients need cannot be met due to persistent donor shortage. To meet the needs, liver tissue engineers made their efforts in both therapeutic and diagnosis approaches, namely, extracorporeal bio-artificial liver devices and tissue-engineered constructs as therapeutic approaches and hepatic drug testing for diagnosis uses:

1): Bio-artificial Liver Assistant Devices. The generated interest of bio-artificial liver device (BALD) is to develop a system in which patient plasma is circulated extra-corporeally through a bioreactor that houses metabolically active liver cells (hepatocytes) sandwiched between artificial plates or capillaries to support a failing liver in the same way that dialysis supports the failing kidney [21]. It requires keeping a large amount of functional cells inside the engineered devices to fulfill the liver functions outside of human body [22-25]. Those devices include hollow fiber devices, flat plate systems, perfusion beds, and suspension reactors, which have shown encouraging results but have been difficult to implement in the clinical setting. The most common bio-artificial liver device design incorporates hepatocytes in hollow fiber cartridges. Hollow fiber

membranes provide a scaffold for cell attachment and immuno-isolation, and are well characterized in a clinical setting, but may not provide adequate nutrient transport or the proper environmental cues for long-term hepatocytes stabilization. Flat plate or monolayer bioreactors have been showed to be able to offer better control of hepatocytes microenvironment, but not ideal for scale up [26,27]. There are also many other designs, which use perfusion environment or scaffolds to promote three-dimensional architecture and minimize transport barriers. However, it may be difficult to provide uniform perfusion of the packing matrix; and cells can be exposed to damaging shear forces [27,28]. Encapsulated suspended cells or spheroid aggregates have been incorporated in perfusion systems that would be simple to scale up, but are limited in their ability to stabilize cells [27,29].

Although many devices include a combination of convective and diffusion transport flow environment, mass transfer limitations of key nutrients to and from the cellular compartment still exist due to diffusion resistance [30]. Barriers to diffusive transport, in those cases, include membranes, collagen gels, and nonviable cells. Apart from culture system consideration, one of the main challenges in BLAD design is to provide a proper microenvironment for primary hepatocytes to maintain liver-specific function, which is absent in many current device designs [31]. One of the essential requirements for BLAD is to recapture the *in vivo* liver structure *in vitro*. In attempts to improve the hepatocytes microenvironment, investigators have used micro-carriers; gel entrapment, both intra-luminal and in the extra-capillary space; multi-compartment interwoven fibers; and multi-coaxial configurations [27]. However, much more efforts are needed in the design and

optimization of culture models that are able to stabilize hepatocytes with cell–cell interactions, cell–matrix interactions, and chemical cues.

2): Tissue engineering constructs. Although the approach remains largely experimental and must overcome a number of significant hurdles before it will become a viable clinical modality, tissue engineering of implantable cellular constructs become more and more attractive as an emerging strategy for liver disease. Similar to cell transplantation, hepatocytes are transplanted to perform liver functions; however, due to anchorage dependent property of hepatocytes, it needs to be immobilized on scaffolds, encapsulated in aggregates, or cultured *ex vivo* to form liver "organoids" and surgically transplanted [32]. Most of proposed constructs need to utilize scaffolds of various chemical compositions, both synthetic and biological compositions including biodegradable polyesters, polysaccharides etc [33-35] and hyaluronic acid, collagen etc [36-38] respectively. It has been reported that scaffold architecture and chemistry play essential roles in hepatocytes survival, morphogenesis, and function. Many studies showed an advantage of three dimensional scaffold architectures over the two-dimensional; and functionality of implantable cellular constructs may be improved by incorporating cell culture strategies that promote three-dimensional conformations and maintain hepatocytes polarity [39]. Some proposed constructs use the encapsulation schemes; and hepatocytes have been encapsulated in fibers, alginate and alginate–polylysine composites to promote cell aggregation and liver-specific function as well as provide immuno-isolation [40-42]. Encapsulation strategies for many different cell types, including highly metabolic hepatocytes, face a classic dilemma between restricting transport of immuno-modulators while maximizing transport of nutrients and desired cell

products. Also, spherical hepatocytes aggregates, heterospheroids of hepatocytes and nonparenchymal cells, and cocultures formed on *in vitro* templates have been proposed as tissue organoids for implantation [43-45]. While still in laboratory trying, hepatocytes have been implanted in many sites including the peritoneal cavity and mesentery, as well as the spleen, liver, pancreas, and subcutaneous tissues [46, 47].

Despite significant progress made *in vitro*, tissue engineering liver construct faces many challenges, mainly limited by cell sourcing, immune rejection, and long-term viability maintenance with additional issues such like transport limitations, the instability of the hepatocytes phenotype when isolated from the hepatic microenvironment and the ability for tissue structures to reorganize over time. Accordingly, fundamental research in tissue engineering has been in the metabolic requirements of hepatocytes during seeding and in early stages of implantation, design of biomaterials to improve angiogenesis, effects of hepatocytes microenvironment on phenotypic stability (by manipulating soluble signals, cell–substrate interactions, and cell–cell interactions), and morphogenesis of hepatocytes structures in pure cultures[48,49]. Most importantly, none of the current proposed constructs incorporates in their designs excretory function corresponding to the biliary system, although studies indicate that morphogenesis can be achieved *in vitro*. In the future, advances in developmental biology will likely complement “brute force” strategies to replicate the exquisite micro-architecture of the liver and its myriad functions. For example, soluble (fibroblast growth factor) and unidentified insoluble factors have been identified in differentiation of the endoderm along the hepatic lineage as well as in branching morphogenesis of the primitive kidney [50].

3): Hepatic-drug testing: The liver is the most important organ concerning the biotransformation of xenobiotics. It plays a major role in the conversion of lipophilic into hydrophilic compounds which can be readily excreted. The metabolism of chemicals usually involves two enzymatic steps commonly referred to as phase I and phase II [51, 52]. Phase I metabolism is ensured mostly by cytochrome P450 (CYP) monooxygenases such as EROD (ethoxyresorufin-O-deethylase, CYP 1A2) and ECOD (ethoxycoumarin-O-deethylase, CYP 2B6). The oxidized metabolite is further conjugated in phase II by UGTs (UDP-glucuronosyltransferases), STs (sulfotransferases), and GSTs (glutathione-S-transferases). The different enzymes necessary for the biotransformation are easy to induce by a high or long substrate supply or an inducing agent. Therefore, the metabolism and consequently the influence of drugs can be essentially affected.

Because of the important roles of liver, i*n vitro* liver preparations are increasingly used for the study of hepatotoxicity of chemicals. In recent years, various *in vitro* models were developed with their actual advantages and limitations defined. The sandwich configuration, liver slices, and 2D hepatocytes culture system, appear to be the most common *in vitro* systems used, as liver-specific functions and responsiveness to inducers are retained either for a few days or several weeks depending on culture conditions [53]. Maintenance of phase I and phase II xenobiotic metabolizing enzyme activities have been proved in those systems; and those systems allows various chemical investigations to be performed, including determination of kinetic parameters, metabolic profile, interspecies comparison, inhibition and induction effects, and drug-drug interactions [54,55]. *In vitro* liver cell models also have various applications in toxicology: screening of cytotoxic and genotoxic compounds, evaluation of chemoprotective agents, and determination of

characteristic liver lesions and associated biochemical mechanisms induced by toxic compounds. Extrapolation of the results to the *in vivo* situation remains a matter of debate.

Recently, hepatic transport processes have been recognized as important determinants of drug disposition. Therefore, it is not surprising that characterization of the hepatic transport and biliary excretion properties of potential drug candidates is an important part of the drug development process [56]. Such information also is useful in understanding alterations in the hepatobiliary disposition of compounds due to drug interactions or disease states. Basolateral transport systems are responsible for translocating molecules across the sinusoidal membrane, whereas active canalicular transport systems are responsible for the biliary excretion of drugs and metabolites [57]. Several transport proteins involved in basolateral transport have been identified including the Na+-taurocholate co-transporting polypeptide, organic anion transporting polypeptides, multidrug resistance–associated proteins and organic anion and cation transporters. Canalicular transport is mediated predominantly via P-glycoprotein, MRP2, the bile salt export pump and the breast cancer resistance protein. The development of *in vitro* techniques to examine hepatic drug transport processes in human liver will provide important insights regarding hepatobiliary drug disposition in humans. Elucidating the mechanisms involved in hepatic drug transport, defining patient-specific factors that affect transporter function, and characterizing how xenobiotic interactions may alter these processes, are fundamental to our knowledge of how the liver disposes of endogenous and exogenous compounds and are prerequisites to exploiting these processes to achieve desirable clinical outcomes.

1.1.3 Liver physiology and general requirement of engineered *in vitro* models

As stated in the above section, these therapies share a general requirement for adequate cell culture environment and stability of liver-specific functions. The success of cellular therapies ultimately depends on the stability of the hepatocytes phenotype and its regulation by micro-environmental cues. Primary hepatocytes are anchorage dependent and notoriously difficult to maintain *in vitro*. Freshly isolated cells rapidly lose adult liver morphology and differentiated functions when cultured in suspension. For years, investigators have developed culture models based on features of liver architecture to recapitulate the complex hepatocytes microenvironment.

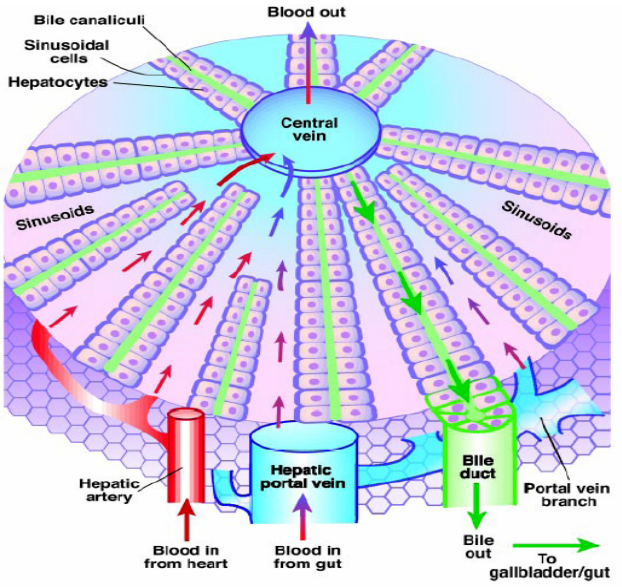


Figure 1 **Celluar architecture of the liver [19].** Liver epithelial cells called hepatocytes are arranged in cords between the capillaries (sinusoids) of the liver. Oxygenated blood enters the liver from the heart via the hepatic artery and from the gut via the hepatic portal vein, mixes in the sinusoids, and drains via the hepatic central vein back to the heart. Sinusoidal cells including endothelial cells, Kupffer cells, and stellate cells line the sinusoids, thus separating hepatocytes from blood. Picture use with author's permission.

The *in vivo* microenvironment may provide a point of reference in engineering culture environments for hepatocytes *in vitro*. Hepatocytes *in vivo*, are exposed to a variety of microenvironmental cues which are in contact with different polarized domains of the plasma membrane associated with distinct functions [61] (Figure 1): the sinusoidal (basal) region specialized for the exchange of metabolites is in contact with loose ECM and sinusoidal plasma flow in the space of Disse; the intercellular (lateral) domain whose tight junctions constitute the canaliculo-

sinusoidal barrier are the sites in which hepatocytes form tight cell-cell adhesions with each other; the canalicular (apical) surface of hepatocytes highly specialized for the secretion of bile acid and detoxification products faces a lumen which delivers bile to the bile ductules. All of these factors, together with their interactions with non-parenchymal cells and the exposure to acinar gradients of nutrients and xenobiotics, may work cooperatively *in vivo* to supply a microenvironment which allows hepatocytes to maintain their polarized morphology and functions, but ceases to operate when hepatocytes are separated from their native environment.

Based on the understanding of basic liver micro-environment, the successful *in vitro* models need to recapitulate the features of complex hepatocytes microenvironment [31] to achieve: 1): Stabilization and maintenance of various liver specific functions. 2): Re-establishment of liver functional structures such like polarized structures with active bile excretion ability. To reach these aims, there are a few features that must be incorporated or considered in the development of *in vitro* culture models.

1): Cell-matrix interactions, The matrix used for liver engineering includes natural ECMs (such like collagen, Matrigel^{TM}, Biomatrix, laminin, fibronectin) and synthetic ECMs [such like poly-lactic-co-glycolic acid (PLGA) and micro-carriers].The major function of the matrix is to induce three-dimensional states in cells, essential for achieving ideal cellular phenotype and functions. It has been shown that alterations in both the composition and topology of the ECM have been shown to affect hepatocytes function [62-64]. For examples, collagen enhanced hepatocytes differentiation over fibronectin, while Matrigel^{TM}, containing primarily laminin, collagen type IV, heparan sulfate proteoglycan and entactin , maintained higher levels of mRNAs encoding albumin and

several P450 enzymes compared with gelled collagen [65]. Noteworthy, the sandwich configuration, which mimics the matrix configuration in the Space of Disse by entrapping cells between two layers of collagen gel, enhanced and maintained albumin secretion for up to 6 weeks in culture, better than cells in a single layer of collagen gel [66, 67].

2): Cell-cell interactions. Cell-cell interactions, including homotypics interaction between same cell type and hetertypic interactions between two different cell types, are crucial to the function of several organ systems. By restoration of homotypic cell–cell interactions, the hepatocytes spheroids and aggregates formed on non-adherent substrates have been reported to promote the formation of bile canaliculi, gap junctions, tight junctions, and help in stabilizing the primary hepatocytes phenotype [68]. A common feature for hetertypic cell interaction is the interaction of parenchymal cells with nonparenchymal neighbors resulting in the modulation of migration, cell growth and differentiation. Co-culture of parenchymal cells with nonparenchymal have been shown, to varying degrees, to induce phenotypic stability of hepatocytes for up to months in culture. These heterotypic interactions are thought to present a highly conserved signal that greatly augments liver-specific functions.

3): Soluble factors, such as hormones and chemical supplements [71,72]. Normally, the soluble signals have a rapid turnover to activate transduction processes that induce a specific physiologic process such like growth or expression of tissue specific genes. The effect of a soluble factor is entirely dependent, both qualitatively and quantitatively, on the matrix chemistry associated with the cell. Most of those soluble factors can be used to help stabilize hepatocytes morphology and regulate functions.

4): Flow environment. A positive effect of flow environment has been proposed in *in vitro* hepatocyte culture. Hepatocytes have been cultured in suspension, perfused scaffolds and flat plate bioreactors [73]. These have not only increased hepatocytes viability by efficient oxygenation and mass transfer of nutrients and waste products, but have also been reported to enhance cell function and tissue morphogenesis.

1.1.4 *In vitro* culture models for liver tissue engineering

Based on the general requirement of *in vitro* models in liver tissue engineering, for years, investigators have developed culture models based on features of liver architecture to recapitulate the complex hepatocytes microenvironment, ranging from simple monolayer culture to spheroids culture, to sandwich culture and co-culture system and more sophisticated 3-D cultures [74-76].

Hepatocytes cultured as a 2D monolayer attached tightly to either plastic or ECM proteins such as collagen I and laminin, showed deteriorating spreading morphology with relatively low liver-specific function and nearly no native *in vivo* liver-like polarized structure;

Improved spheroid culture configuration is developed based on the observation that self-assembled spherical aggregates of isolated primary hepatocytes have been obtained on numerous moderately-adhesive substrata comprised of natural matrices such as proteoglycan fraction from liver reticulin fibers, agarose, rigid extracellular matrix at low concentration like Matrigel, laminin, fibronectin or collagen type I, and artificially synthetic matrices such as positively charged or galactosylated substrata [77-80]. Hepatocytes spheroids with naturally formed 3D architecture showed associated cell-cell/

cell-matrix connectivity and ideal liver-specific functions, membrane polarities and liver ultra-structures. However, the usefulness of 3D hepatocytes spheroids in applications is limited due to the poor mass transport of nutrients, oxygen, xenobiotics and metabolites into and from the core of these large cellular aggregates [81]. Cell loss is also a critical issue in forming and maintaining these spheroids in applications due to the poor adhesion of spheroids on the substratum [82].

Many groups have shown that hepatocytes can survive for long periods and maintain specific functions when they are cocultured with other cell types, such as nonparenchymal liver cells (NPCs) [69]. It was previously reported that formation of multicellular spheroids consisting of hepatocytes and NPC in a hierarchical co-culture, in which both cell-types were separated by a collagen layer, was very effective for the maintenance of liver functions, such as albumin secretion, urea synthesis and induction of tyrosine aminotransferase [83]. However, due to the system complexity and the lacking of valid mechanisms regarding cell-cell interaction and various soluble factors involved, these approaches, still have a long way to reach the practical uses. Also, the native liver-like structure, such as polarized structure, is hard to form due to the uncontrollable seeding methods.

Sandwich culture, has been recognized as one of the most promising models currently available to impact both in the studies of liver physiology/toxicology and developments of technologies related to cell transplantation and hepatocytes bioreactors [84]. Primary hepatocytes culture in sandwich configuration, formed by overlay of second layer of ECMs support on monolayer cells cultured on single surface, captured the essential characteristics of liver disse, induced the re-establishment of polarity structure with the

maintenance of myriad of enhanced liver specific functions for at least several weeks [85]. These results were first obtained using type I collagen, and more recently similar results have been reported using an overlay of matrix extracted from Engelbreth-Holm-Swarm (EHS) tumor grown in mice. In current sandwich culture practice, hepatocytes maintained on collagen-coated matrix are overlaid with a second layer of collagen matrix after one day of monolayer culture [86]. Further application of such conventional sandwich was mainly limited by complex compositions of natural ECMs that have not been clearly identified, batch-to-batch variations of natural ECMs and the transport barriers caused by the introduction of top ECM layer, which can slow down the exchange of nutrients, products, and chemical signals with the bulk of the medium.

1.2 Primary hepatocytes in sandwich culture

1.2.1 Potential applications of sandwich culture in liver tissue engineering

In terms of application values for these in vitro models, sandwich configuration is an ideal model with the stable functional maintenance and re-establishment of polarity structure of primary hepatocytes cultured in between; and has proved its values in studies of hepatic tissue physiology and toxicology: to characterize the dynamics of induction and functional properties of liver-specific cytochrome P450 systems and to examine the temporal aspects of the cytokine-induced response, as well as bile excretion ability which are important in hepatic drug deposition and drug-drug interaction.

Current BLAD devices suffer from the limited excretory functions to sustain themselves when exposed to toxins in patients' blood. Hepatocytes in BLAD normally cannot last for 1 day when exposed to patients' blood due to inefficient secretion of toxins out of cell

body via several of transporters inside and formation of bile canaliculi outside. With high excretory function and polarity re-establishing, we hypothesis that sandwich configuration can serve as a potential culture configuration to be incorporated in next generation of BLAD applications. Therefore, the author mainly focused on the sandwich models for hepatocytes culture.

1.2.2 Polarity genesis of hepatocytes in sandwich culture

To hepatocytes monolayer on ECMs, the overlay of ECMs establishes a sandwich configuration resembling that found in the liver (i.e., where hepatocytes are generally bounded by ECM at each of their opposite basolateral membrane domains). It was reported that hepatocytes remained as a monolayer but underwent major changes at the intracellular level that culminated in the formation of a 2-dimensional, multicellular network with a functional bile canalicular network reminiscent of the liver plate [85].

Recent studies indicate that the configuration of ECM has a dramatic and reversible effect on the organization and expression of cytoskeletal proteins in cultured primary hepatocytes [87]. Microtubules in hepatocytes cultured on a single collagen gel were organized into long parallel arrays extending out to the cell periphery, while those in sandwiched hepatocytes were organized into a dense meshwork. F-actin in hepatocytes cultured in a double collagen gel was concentrated under the plasma membrane in regions of contact with neighboring cells, similar to what was observed in *in vivo* distribution. In contrast, hepatocytes cultured on a single gel exhibited random F-actin distribution with stress fibers on the ventral surface in contact with the substrate.

It has been demonstrated that a contiguous network of bile canaliculi was formed throughout the entire sandwich culture [67]. After overlay, bile canalicular formation initiates as punctate lumina between adjacent hepatocytes. These sites propagate along the cell borders and eventually fuse into a complete network. Normal bile canalicular function and integrity as evidenced by carboxy-fluorescein retention were observed within 3-4 days after overlay [88], while without the collagen overlay, canalicular formation is more variable in rate and extent, and eventually ceases when cells begin to detach and die (5 to *7* days after seeding). Noteworthy, hepatocytes on a type I collagen substrate and overlaid with EHS matrix form a similar bile canalicular network. The formation of bile canaliculi occurs in concert with changes in the distribution of microtubules and microfilaments [67], with a marked accumulation of these cytoskeletal proteins occurred at sites of canaliculi generation. The roles of cytoskeleton in bile canaliculi have been investigated in many cases using disrupting reagents. Microtubule-disrupting agents (colchicine, nocodazole) prevent the normal accumulation of actin at the cell margins and inhibit canaliculi formation. Microfilament-perturbing agents (cytochalasin D, phalloidin) have little effect on the initiation of canalicular development or on the distribution of microtubules, but prevent the normal elongation and proliferation of the nascent canaliculi into a network. Once bile canalicular structures are nearly complete, actin microfilaments appear to be primarily associated with them, whereas microtubules become more uniformly distributed throughout the cell. Treatment with microtubule-disrupting agents at that time do affect the integrity of preformed canaliculi, but microfilament-perturbing agents cause a marked dilation of the lumen.

Staining of collagen sandwiched hepatocytes with antibodies specific to several

basolateral and apical markers (glucose transporter, Na+, KtATPase, aminopeptidase N, dipeptidylpeptidase IV) as well as the cell-cell adhesion cadherin reveal a distribution identical to liver, which suggests that this culture configuration preserves the polarized phenotype of normal hepatocytes. In the liver, the efficiency of passive diffusion of xenobiotics across the canalicular membrane is poor; instead, several active transporters exist on this membrane that efflux a variety of endogenous and exogenous materials from the cell into the bile canaliculus (BC). Two canalicular active transporters have been demonstrated to function well in sandwich configuration and contribute to the efflux of xenobiotics, the multidrug resistance-associated protein (Mrp2) and P-glycoprotein (P-gp) [89,90]. Mrp2 is a major transporter of bilirubin, glucuronide- and glutathione-conjugates, and other organic anions from liver into bile, while the P-gp facilitates the excretion of exogenous organic cations and a wide variety of drugs, such as alkaloids and anthracyclines into bile. Improved hepatocytes repolarization may improve the functional activity of these canalicular transporters which in turn facilitate the efficient excretion of waste products into a bile canalicular network which is structurally separate from the cells. In a review on new perspectives in generating epithelial cell polarity, A model has been proposed in which cell-matrix and cell-cell adhesion generate membrane asymmetry which orientate the apico-basal axis of polarity relative to the external cues [91]. Expression of connexin 32 was also reported with comparing of hepatocytes cultured on a single collagen gel with similar cultures overlaid with EHS matrix [92]. In the recent studies, canalicular localization of ‘y-glutamyltranspeptidase, Mg2+_ATPase, and actin have also been reported [89]

1.2.3 Functional maintenance of hepatocytes in sandwich culture

Isolated hepatocytes placed in a type I collagen sandwich exhibit a gradual increase in the expression of liver-specific function during the first week in culture; conversely, the same cells placed on a single ECM-coated surface progressively stop expressing these functions and loose viability [84]. Beyond the first week, the expression of liver-specific functions in the type I collagen sandwich is stable over several weeks. The effect of collagen overlay is best illustrated when a week-old culture of hepatocytes on a simple collagen gel that have already lost much of their normal phenotype can be “rescued” by addition of a collagen overlay to produce a stable, functional culture [85], suggesting that there is a sensitive dynamic relationship between the ECM configuration and the intracellular events that determine hepatocytes morphology and liver-specific function. Although the collagen overlay causes several changes in hepatocytes morphology and function that occur over different time scales, these various changes have not yet been causally related.

After isolation, when hepatocytes are placed in culture, induction of albumin synthesis that parallels an increase in albumin mRNA levels occurs during the first 7 days post-overlay [93]. Nuclear run-off assays showed that higher transcriptional activity was responsible for the higher level of albumin mRNA in hepatocytes cultured in the sandwich system compared to the single gel system. In addition, a concomitant increase in the size of polyribosomes associated with albumin mRNA was found. Furthermore, the secretion kinetics of synthesized albumin was assessed with pulse-chase experiments. In hepatocytes 1 day post-isolation, the transit time of secretion was roughly the same as in liver, suggesting that impaired transport or internal degradation were not responsible for

the low initial rate of albumin secretion of cultured hepatocytes. These results suggest that the collagen overlay mediated its enhancing effect on albumin secretion primarily via an increase in albumin mRNA levels. This increase most likely resulted from an increase in the rate of transcription of the albumin gene. Apart form albumin synthesis, urea production and phase I and phase II metabolites have also been reported to have a tremendously increase in the sandwich configurations [94].

1.2.4 Inherent mass transfer barrier in sandwich configuration

Hepatocytes sandwich culture involves culturing cells between two layers of extra-cellular matrix support on solid surfaces. The cells are generally seeded onto a collagen-coated polystyrene or glass surfaces and sandwiched by another layer of the collagen on porous membranes so as to allow nutrient access from the culture medium above the sandwich assembly. The two layers of support divide the sandwich culture assembly into two environments, the extra-sandwich and the intra-sandwich environments. The former is the well-controlled environment defined by the culture medium, and the latter is the cell-containing environment between the two layers of support. The barrier will impede the mass transfer between the two environments, resulting in the uneven distribution of nutrients and metabolites in both environments, most obviously, the accumulation of metabolites, especially macromolecules with low diffusion coefficients such as albumin.

Albumin, a globular protein with a MW of 69,000, is synthesized in the liver and catabolized by all metabolically active tissues. It can be a useful indicator of liver functions and serve as a carrier protein for many organic substances such as unconjugated bilirubin, and bile acids etc [95, 96]. As the most abundant molecules with a relatively

lower diffusion coefficient, albumin can also be a good model molecule to indicate the mass transport properties of metabolites in sandwich culture. Previous studies reported that free diffusion coefficients of albumin are in the order of 10^{-11} $m^2 \cdot S^{-1}$ [97]. But in gels, diffusion coefficient of albumin is much lower than in free solution, due to the effect of hydrodynamic and steric factors. The diffusion coefficients of albumin through the collagen membrane and other gel matrix have been observed, ranging from 10^{-15} to 10^{-12} $m^2 \cdot S^{-1}$ [98,99]. A similar phenomenon was also observed that significantly more bilirubin-glucuronides were kept in the intra-sandwich environment than in the medium in 96h convectional sandwich culture of both human and rat hepatocytes.

1.3 Roles of flow environment in facilitation of the mass transfer efficacy

1.3.1 Bioreactor in tissue engineering applications

Major obstacles to the generation of functional tissues and their widespread clinical use are related to a limited understanding and ability in designing specific physicochemical culture parameters on tissue development. By enabling reproducible and controlled changes of specific environmental factors, bioreactor systems provide both the technological means to reveal fundamental mechanisms of cell response in artificial environment, and the potential to improve the quality of engineered tissues.

Bioreactors are generally defined as devices in which biological and/or biochemical processes develop under closely monitored and tightly controlled environmental and operating conditions (e.g. pH, temperature, pressure, nutrient supply and waste removal). In tissue engineering approaches, the role of bioreactors in processes is key for the ex

vivo engineering of 3D tissues based on cells and scaffolds, including cell seeding on ECM support, nutrition of cells in the resulting constructs, and mechanical stimulation of the developing tissues, particularly with the ability to control over environmental conditions such as flow environment, mechanical force to optimize the culture environment.

1.3.2 Enhancement of mass transfer efficacy by flow environment in bioreactors

It has long been known that the supply of oxygen and soluble nutrients and secretion of big metabolites become critically limited for many *in vitro* culture models such as spheroid culture and sandwich culture. The consequence of such a limitation is exemplified by early studies showing that cellular spheroids larger than 1 mm in diameter generally contain a hypoxic, necrotic center, surrounded by a rim of viable cells [100]. Similar observations were reported for different cell types cultured on 3D scaffolds under static conditions. For example, glycosaminoglycan (GAG) deposition by chondrocytes cultured on poly(glycolic acid) meshes was poor in the central part of the constructs (400 mm from the outer surface), and deposition of mineralized matrix by stromal osteoblasts cultured into poly(DL-lacticco-glycolic acid) foams reached a maximum penetration depth of 240 mm from the top surface [101]. Since engineered constructs should be at least a few mm in size to serve as grafts for tissue replacement, mass-transfer limitations represent one of the greatest challenges to be addressed.

External mass-transfer limitations can be reduced by culturing constructs in a stirred flask. As one of the most basic bioreactors, the stirred flask induces mixing of oxygen and

nutrients throughout the medium and reduces the concentration boundary layer at the construct surface. Culture of bovine chondrocytes on poly(glycolic acid) non-woven meshes in a stirred flask induced an increase in both the synthesis of GAG and the fractions of GAG accumulated within the central construct regions [102]. However, this approach will probably cause the turbulent eddies generated within the stirred-flask bioreactor. A dynamic laminar flow generated by a rotating fluid environment is an alternative and efficient way to reduce diffusion limitations of nutrients and wastes while producing low levels of shear. The good efficacy of rotating wall vessel (RWV) bioreactors for the generation of tissue equivalents has been demonstrated using chondrocytes, cardiac cells and various tumor cells [103]. After a few weeks of cultivation in the RWVs, cartilaginous constructs had biochemical and biomechanical properties superior to those of static or stirred-flask cultures, comparable to those of native cartilage, whereas cardiac tissue constructs consisted of elongated cells that contracted spontaneously and synchronously [104]. Prostate and melanoma cancer-derived cells cultured in RWV bioreactors had 3D structures that reflected the cellular architecture and heterogeneous composition of the tumor site *in vivo*. On the basis of these studies, it was proposed that the RWV bioreactor would support the engineering of tissues and organoids as *in vitro* model systems of tissue development and function [105]. Bioreactors that perfuse medium either through or around semi-permeable hollow fibers have been used successfully to maintain the function of highly metabolic cells (e.g. hepatocytes) by increasing the mass transport of nutrients and oxygen. This concept has been extended to engineered tissues by perfusing culture medium directly through the pores of the cell-seeded 3D scaffold, thereby reducing mass transfer limitations both at

the construct periphery and within its internal pores. Direct perfusion bioreactors have been shown to enhance growth (differentiation and mineralized matrix deposition by bone cells), proliferation of human oral keratinocytes and albumin synthesis rates by hepatocytes [106-110]. When incorporated into a bioreactor design, direct perfusion can thus be used as a valuable tool for enhancing cell survival, growth and function. However, the effects of direct perfusion can be highly dependent on the medium flow-rate and the maturation stage of the constructs. Therefore, optimizing a perfusion bioreactor for the engineering of a 3D tissue must address a careful balance between the mass transfer of nutrients to and waste products from cells, the retention of newly synthesized extracellular matrix components within the construct, and the fluid induced shear stresses within the scaffold pores.

Currently, the optimal flow conditions of a bioreactor were determined through a trial-and-error approach. Researches, by manipulating flow environment, aim to control the mass transfer behavior of aimed nutrients or metabolites from a diffusion dominated process to a convection dominated process.

1.3.3 Current practices of bioreactors in liver tissue engineering

A positive influence of the flow environment in hepatocytes culture has been widely accepted for improving mass transfer [111]. The effects of flow environment on hepatocytes functions and mass transfer behavior have been validated in various bioreactors such as flat-plate bioreactor and grooved bioreactor [112,113]. Previous studies of perfusion culture involving hollow fiber bioreactors have demonstrated that the transport of nutrients and metabolites across the hollow fiber membrane can be regulated

from the slow diffusion-dominated process to the fast convection-dominated process by manipulating the flow rates of the perfusate [114].

In sandwich culture, perfusion bioreactors may assist the mass transfer across the barriers in a sandwich construct. The perfusion rates vary a lot in different bioreactor configurations, even all of them are in the lower range of flow rate compared with flow rates used in other tissue engineering. This is most likely because hepatocytes are highly shearing force- sensitive cells. High flow rates in a convection-dominated process might yield efficient mass transfer but might be detrimental to the functions of highly sensitive hepatocytes. Previous studies showed that shear stress could damage the cells under high flow conditions and excessive mass exchange could induce culture conditions as well as losing of metabolites essential for cell maintenance [113]. Therefore, it will be important to carefully control the flow conditions in perfusion sandwich culture such that both efficient mass transfer and minimal cell damage can be achieved to maintain hepatocytes functions.

1.4 Synthetic polymer ECMs in liver tissue engineering

Extracellular matrix (ECM) plays important roles in tissue engineering because cellular growth and differentiation, in the two-dimensional cell culture as well as in the three-dimensional space of the developing organism, require ECM with which the cells can interact. Especially, the bioartificial liver assistant device or regeneration of the liver-tissue substitutes for liver tissue engineering requires a suitable ECM for hepatocytes culture because hepatocytes are anchorage-dependent cells and are highly sensitive to the ECM milieu for the maintenance of their viability and differentiated functions [115-116].

The use of polymeric materials with proper surface modification as synthetic ECMs lead to novel approaches in tissue engineering applications with controllable matrix properties and cellular responses. With functional groups modified on, polymers substitute the natural ECM for many functions, which can organize cells into a three-dimensional architecture, providing mechanical integrity to the new tissue and a space for the diffusion of nutrients to and metabolites from the cell. A variety of synthetic polymeric substrata have been employed for hepatocytes culture (e.g. plastic surfaces or membranes coated with extracellular matrix proteins such as laminin, fibronectin or conjugated with cell adhesion peptides, such as Arg-Gly-Asp (RGD) and Tyr-Ile-Gly-Ser-Arg (YIGSR) or galactose [117-119].

1.4.1 Galactose-carrying synthetic ECMs

Galactose-carrying synthetic ECMs derived from synthetic polymers and natural polymers bind hepatocytes through a receptor-mediated mechanism, resulting in enhanced hepatocytes functions. Attachment and functions of hepatocytes were affected by physico-chemical properties including ECM geometry as well as the type, density and orientation of galactose. Also, cellular environment, medium composition and dynamic culture system influenced liver-specific functions of hepatocytes beside ECMs.

The first galactose-carrying ECMs is poly (acryl amide) containing covalently immobilized galactose groups among the synthetic polymers [120]. It was reported that rat hepatocytes were bound to a specific sugar in a Ca^{2+}-dependent manner; and cell binding to these surfaces was specifically inhibited by asialo-orosomucoid. Rat hepatic lectins found on the hepatocytes cell surface mediated adhesion of isolated primary rat

hepatocytes to galactose derivatized poly acrylamide gels [121].

Galactose-derivatized polystyrene (PS), poly (*N*-*p*-vinylbenzyl-4-*o*-*β*-d-galactopyranosyl-d-gluconamide)(PVLA), as a synthetic polymer, has been reported as an excellent synthetic ECM to guide hepatocytes adhesion through the unique ASGPR–galactose interaction, although ASGPR is a non-adhesion cell surface receptor [122]. The synthesis of PVLA is simple, protection of the hydroxyl groups of oligosaccharides is not required, and the yield of each step is high. In addition to galactose-specific molecular recognition between ASGPR of hepatocytes and highly concentrated galactose moieties along the polymer chains; the round morphology of hepatocytes on PVLA was found to trigger the formation of multi-cellular aggregates in the presence of epidermal growth factor (EGF), which is the first report of spheroid formation of hepatocytes through receptor-mediated mechanism, leading to enhanced cell functions [123].

It has also been reported that hepatocytes cultured on galactose-modified star poly(ethylene oxide) hydrogels exhibited a sugar-specific adhesion to the modified gels, adhering to gels bearing galactose but not glucose [124]; and cell spreading was observed on low concentrations of immobilized ligands. Galactose ligands have been successfully immobilized on acrylic acid graft-copolymerized poly(ethylene terephthalate)(PET) film by plasma pretreatment [125]. Certain manner of hepatocytes' behaviors also been characterized on the surface topology on lactose-carrying styrene (VLA) dishes using plasma glow discharge followed by the graft polymerization of VLA [126]. Hepatocytes cultured on the galactosylated surface exhibited good attachment and promoted spheroid formation of the attached cells; and the albumin as well as urea synthesis of hepatocytes cultured on the surface was higher than that on the collagen-modified PET substrates.

New approaches have been focused on coupling galactose ligands on silica surface to get of the actual contact mechanics and adhesion strength of hepatocytes during two-dimensional cell spreading.

1.4.2 RGD motif containing synthetic ECMs

In 1987, the tripeptide RGD was identified to be the cell-adhesion sequence in fibronectin and other cell-adhesion proteins. This discovery enabled systematic engineering of surfaces that either promoted or rejected cell adhesion. RGD can bind to integrins and those that bind to RGD alone can regulate cellular functions antagonistically [117]. Hepatocytes anchor tightly to RGD modified substrata, and exhibit extended and spread cell morphology, with low levels of liver-specific functions likely due to hepatocytes de-differentiation [127]; and RGD-integrin interactions have been shown to be strong enough to induce downstream signaling pathway to cause the redistribution of the cytoskeleton, formation of focal adhesion complex and enhancement of cell-cell interaction in many studies [128].

1.5 Project outline

Current tissue engineering approaches for various medical solutions share a general requirement for adequate in-vitro models with stable liver-specific functions and functional structure features. Sandwich configuration, by culturing hepatocytes between two layers of ECM supports, is ideal for reestablishing cell polarity and maintaining various liver specific functions with high potential for various liver engineering applications. However, current sandwich configurations based on natural ECMs such as collagen type I and matrigel suffer from inherent mass transfers barrier imposed by the

two layers of extra-cellular matrices on semi-permeable support, which can slow down the exchange of nutrients, products, and chemical signals with the bulk of the medium. The two layers of support divide the sandwich culture assembly into two environments, the extra-sandwich and the intra-sandwich environments. The former is the well-controlled environment defined by the culture medium; and the latter is the cell-containing environment between the two layers of support. The barrier impedes the mass transfer between the two environments, resulting in the uneven distribution of nutrients and metabolites in both environments.

This study is aimed to explore solutions to address the problem of mass transfer barriers in sandwich configuration by using two different approaches 1): Since a positive influence of the flow environment in hepatocytes culture has been widely accepted for improving mass transfer, it will be possible to manipulate the flow conditions in a perfusion sandwich culture bioreactor such that both efficient mass transfer and minimal cell damage are achieved. Using a current sandwich configuration with collagen coated ECMs support at both side, we try to reach a guideline regarding the choosing of the flow rates in perfusion sandwich culture; and with the aid of proper bioreactor design and flow control, to achieve high mass transfer efficacy in sandwich culture. 2): Use of polymeric materials with proper surface modification as synthetic ECMs to replace the natural ECMs used in sandwich culture such as collagen type I, which generally process a lower diffusion coefficients compared with synthetic ECMs. By conjugating the PET surface with various functional groups such as galactose or RGD motif, we aim to develop a natural ECMs-free sandwich configuration with higher mass transfer efficacy, stable functional maintenance and expression of liver-specific functional structure.

The solutions to inherent mass transfer barriers in sandwich culture and associated

process may represent novel approaches to improve the performance of hepatocytes sandwich culture for relevant applications.

Chapter 2 Materials and Methods

All reagents were purchased from Sigma-Aldrich unless stated otherwise.

2.1 Hepatocytes isolation and culture

Hepatocytes were harvested from male Wistar rats weighing 250-300 g by a two-step *in situ* collagenase perfusion method [134]. Cell viability was determined to be at least 90% by Trypan Blue exclusion assay. The seeding density is about 0.2 million per 9 mm diameter attachment area. Hepatocytes were cultured by utilizing Hepatozyme SFM (Gibco Laboratories, Carlsbad, California), supplemented with 100 μM dexamethasone and 100 μg/ml penicillin/streptomycin.

2.2 Fabricating PET film conjugated with galactose (PET-f-Gal)

Poly acrylic acid (pAA) was grafted onto the PET film surface with a modified protocol for conjugating bioactive ligands [135]. Briefly, PET film was cut into 2cm x 8cm strips and cleaned in ethanol. The air-dried PET strips were subject to argon plasma treatment which was carried out in SAMCO Basic Plasma Kit (SAMCO INTERNATIONAL INC.) operating at a radio frequency of 13.6MHz. Argon was introduced into the chamber in the SAMCO kit at a flow rate of 50ml/min with chamber pressure maintained at 20Pa. Plasma was generated at an electric power of 40W for 1 min. After the plasma treatment, the PET strips were exposed to atmosphere for 10 minutes to promote the formation of surface peroxides and hydroperoxides, which were used for the subsequent UV-induced grafting of pAA. For the UV-treatment, quartz tubes with length of 12cm and diameter of 2.5cm were

fabricated at the Glassware workshop of the Department of Chemistry at NUS. The plasma-treated PET-strip was immersed in 30ml of the aqueous solution containing acrylic acid in the quartz tube. Argon was bubbled through the solution to thoroughly remove oxygen and capped under Argon. The quartz tube was placed in water bath with constant temperature of 28 ^{o}C and then subjected to UV irradiation for 30min using a 400W flood lamp in UV-F 400 unit (Panacol-Elosol GmbH). After grafting, the PET strip was taken out of the tube and washed exhaustively with DI water for 24h to remove the residual homopolymer absorbed on the surface.

pAA-g-PET strips were cut into circular disks with diameter of 15 mm in order to fit into the 24-well microplates. Galactose ligand were conjugated simultaneously using a 'two steps' EDC (1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide Hydrochloride) chemistry. Briefly, at the first step, 100µl of MES buffer (50mM, pH of 5.5) containing 1.5mg EDC and 0.3mg sulfo-NHS were added to each 96-well containing the pAA-g-PET disk to activate the surface carboxylic groups by forming NHS esters. After 2h activation at room temperature, the MES solution was completely removed and replenished with 100µl phosphate buffer (0.1M, pH of 7.4) containing ligands and allowed to react with activated substratum by shaking at 300rpm in a thermomixer (Eppendorf) for 48h at 4 ^{o}C. PET-Gal was fabricated by reaction with AHG ligand respectively. After conjugation of the bioactive ligands, each sample was quenched with 0.5% ethanolamine solution for 15min to block non-specific interactions due to the un-reacted carboxylic groups with the hepatocytes.

2.3 Fabricating PET track-etched membrane conjugated with Galacotose (PET-m-Gal) or RGD (PET-m-RGD)

The PET track-etched membrane was cut into circular disk with diameter of 1.2cm to

fit into minusheet carriers. The membrane disk was pre-treated by oxidization with a solution of KMnO4 (60 g) in 1.2 N H2SO4 (1.5 l) at 60 ^{o}C during 1 h in order to enhance the amount of carboxyl chain-ends displayed on their surface, then rinsed at 20 ^{o}C successively with 6 N HCl (2*10 min) and water (HPLC grade; 3*10 min). 300µl of MES buffer (50mM, pH of 5.5) containing 10mg EDC and 2mg sulfo-NHS were added to each well containing the PET-COOH membrane to activate the carboxylic groups by forming NHS esters. After 2h activation at room temperature, the MES solution was completely removed and replenished with 300µl phosphate buffer (0.1M, pH of 7.4) containing ligands and allowed to react under shaking at 300rpm in a thermomixer (Eppendorf) for 48h at 4 ^{o}C. PET-m-RGD or PET-m-Gal was fabricated by reaction with 0.2mg RGD or 1mg AHG respectively. After conjugation, each sample was blocked with 0.5% ethanolamine solution for 15min to quench non-specific interactions due to the un-reacted carboxylic groups. The microplates containing different substrata were sterilized by soaking with 70% ethanol for 3h and then rinsed 3 times with PBS for cell culture.

2.4 Characterization of PET-RGD and PET-Gal substrata

The graft density of carboxylic groups on the PET films and membranes was determined by a colorimetric method using Toluidine Blue O staining [136]. XPS was used to qualitatively verify the pAAc grafting and ligand conjugation onto the PET. Measurements were made on a VG ESCALAB Mk II spectrometer with a Mg Kα X-ray source (1253.6 e V photons) at a constant retard ratio of 40.

Gal ligands on PET film and membrane were hydrolyzed off the substrata using Acid Hydrolysis Station (C.A.T. GmbH & Co.) in 6N HCl at 110 ^{o}C for 24h under vacuum. The cooled hydrolyzed solution was filtered into a new vial and evaporated under

nitrogen. The hydrolyzed ligands from PET were re-suspended in 50µl DI-water and derivatized using ATTO-TAG™ CBQCA Amine-Derivatization Kit (Molecular Probes) for fluorescence detection after separation on a reverse phase C-18 column in HPLC (Agilent Technology). Optimized mobile phases are: A, Water + 0.1% TFA; B, Acetonitrile + 0.1% TFA. Standard curves were established against soluble Gal ligands.

2.5 Collagen coating and sandwich culture configuration

Forty µl of neutralized Type I Bovine dermal collagen (8 ml collagen, 1 ml 0.1 M NaOH, 1 ml 10xPBS, 6 ml 1xPBS) from Vitrogen, Angiotech BioMaterials Corp. (Palo Alto, CA) was spotted onto 12mm coverslips and 12 mm cellulose paper inserts (Millipore Corp., Billerica, MA) and were then transferred into a 37^{o}C incubator for collagen gelation to occur overnight. Freshly isolated rat hepatocytes were seeded on the collagen-coated coverslips, and after 1 hour of incubation, it was immediately overlaid with collagen-coated cellulose paper which was stabilized using the O-rings on the minusheet carriers (Minucells and minutissue Vertriebs GmbH, Bad Abbach, Germany). After that, the entire configuration was transferred to a 24 well culture dish or perfusion chamber for cell culture.

To form synthetic sandwich configuration, freshly isolated rat hepatocytes were seeded onto different substrata at the seeding density of 0.2 million per 9 mm diameter attachment area. Biaxially oriented poly (ethylene terephthalate) (PET) films of about 100 µm in thickness were purchased from Goodfellow Inc. of Cambridge, U.K. PET track-etched microporous membrane (CycloporeTM) were purchased from Whatman International Ltd (Maidstone, England). This membrane was characterized by a thickness of 22 µm, a pore density of 2.2*10^{7} pores/cm2, and a mean pore

diameter of 1μm. The galactose ligand, 1-O-(6'-aminohexyl)-D-galactopyranoside (AHG, M.W. 279) was synthesized according to the method developed previously [137, 138] and verified by NMR spectrum. RGD peptide (GRGDS) was bought from Peptides International. Hepatocytes on the galactosylated PET film were incubated for 3h for full attachment. When 3D monolayer was formed after one day of culture, another layer of RGD-conjugated PET membrane was overlaid and the sandwich construct were secured using the O-rings on the minusheet carriers. For conventional collagen coating, 40 μl of neutralized Type I Bovine dermal collagen (8 ml collagen, 1 ml 0.1 M NaOH, 1 ml 10xPBS, 6 ml 1xPBS) from Vitrogen, Angiotech BioMaterials Corp. (Palo Alto, CA) was spotted onto 12 mm glass coverslips before being transferred into a 37 ^{o}C incubator overnight for collagen gelation to occur. Hepatocytes in the conventional sandwich culture were incubated for 1h for full attachment and then overlaid with un-gelled collagen after 24 h culture and gelation was allowed to occur at 37 ^{o}C for 3 h before fresh culture medium was added. Hepatocytes were cultured using William's E culture medium supplemented with 1mg/ml BSA, 10ng/ml of EGF, 0.5μg/ml of insulin, 5nM dexamethasone, 50ng/ml linoleic acid, 100units/ml penicillin, and 100μg/ml streptomycin.

2.6 Bioreactor design and perfusion system

The inner dimensions of the flat plate bioreactor, which has three chambers at the bottom part contain the entire sandwich configuration, are of 75×25×1 mm (L×W×H). The chambers inside this polycarbonate bioreactor have a diameter of 13mm and a depth of 3mm which can hold minusheet carriers with sandwich construct tightly. When minusheet carriers were put in, a close space of chamber will be formed between the bottom of the carriers and the bottom of chambers, which is separated

with the main flow environment at the top of sandwich construct by the minusheet carriers. To obtain optimal equilibrium of the pO2 and pCO2 in perfusion cultures, the medium was pumped through long, small-diameter, gas permeable silicone tubes, to allow continuous and optimal exchange of gases.

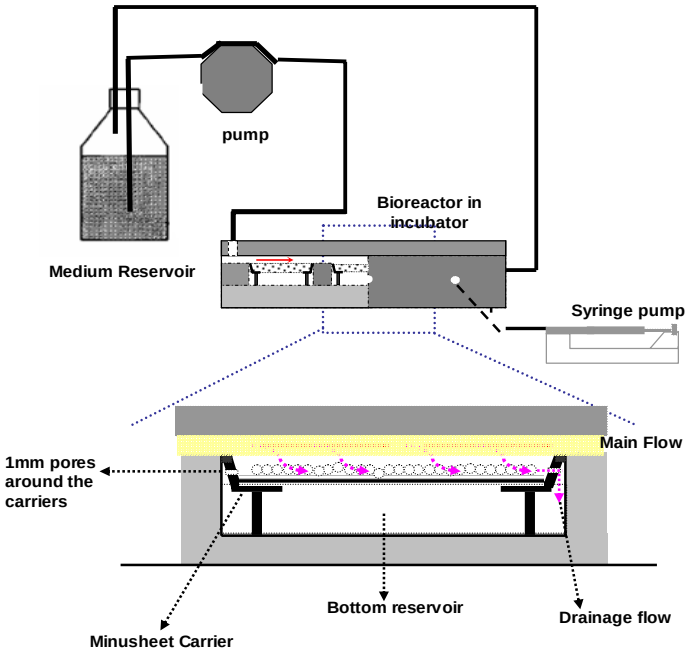


Fig 2.1 Schematic representation of perfusion circuit and separate drainage model for perfusion sandwich culture.

To realize the separate drainage, the intra-sandwich environment of sandwich configuration was linked to the close space of chambers by sixteen small pores (with 1mm diameter) at the side of carrier. Channels were fabricated at the side of each chamber leading to the drainage pump. Two flow paths were established in this system: one is the flow path above the sandwich construct directly. Another is a

branched flow path from the first flow path through intra-sandwich environment and the side pores of the sandwich carrier into the close room of the bioreactor chamber such that the intra-sandwich environment could be regulated directly through controlling the flow rates in this drainage flow path. The schematic drawing of separate drainage and perfusion culture model is shown in Figure 5.1. By controlling the rates of the withdrawal, the cellular environment can be regulated.

2.7 FITC-BSA transport behavior under different flow rates and diffusivity

Measurement of the fluorescein isothiocyanate (FITC)-conjugated BSA behavior through the collagen-coated porous membrane at the top of sandwich construct at different flow rates of 0.1ml/min, 0.5ml/min, 1ml/min, 2ml/min and 5ml/min were based on the same principles as a donor-receptor environment model which was reported previously [139]. The donor environment was formed by clamping between the collagen-coated porous membrane and collagen-coated cover-glass using minusheet carriers. Donor environments were filled with 100 µl of 3.3 mg/ml FITC-BSA in 1xPBS, while receptor environments were being continuously perfused by culture medium. The perfusion medium was collected at different time intervals. The concentrations of FITC-BSA were measured at an excitation and emission wavelength of 490 nm and 525 nm respectively against FITC-BSA standards by employing a microplate reader (Tecan Trading AG, Switzerland).

Measurement of the diffusivity of fluorescein isothiocyanate (FITC)-conjugated BSA was based upon the same donor-receptor environment model and method as the aforementioned one, except that donor environments were filled with 100 µl with a concentration of 0.033 mg/ml FITC-BSA in 1xPBS. The amount of BSA used here is

approximately the same as the amount of albumin produced by cell cultured in this given sandwich configuration.

2.8 FITC-dextran diffusivity measurements

Measurement of the diffusivity of fluorescein isothiocyanate (FITC)-conjugated dextrans of 9.5 kDa, 70 kDa and 150 kDa through the PET membrane and collagen layer were based on the donor-receptor compartment model described above. Briefly, the membrane was clamped between the receptor and donor compartments using minusheet carriers. Donor compartments was filled with 20 µl of 0.2 wt % FITC-dextran in 1xPBS, while receptor compartments were filled with 200 µl of 1xPBS. Samples were taken from the receptor compartment at each hour and replaced with fresh PBS. For the measurement of diffusivity of fluorescein isothiocyanate (FITC)-conjugated dextrans through collagen, 20 µl of 0.2 wt % FITC-dextran in 1xPBS was maintained in glass coverslip and 40 µl of collagen was added at the top. The whole construct was maintained in minucell carriers and incubated for 4h to let collagen gel. Then 200 µl of 1xPBS was added on the receptor compartment and samples were taken from the receptor compartment at each hour and replaced with fresh PBS. The concentrations of FITC-dextran were measured at 490 nm excitation/525 nm emission against FITC-dextran standards using a microplate reader (Tecan Trading AG, Switzerland).

2.9 Biliary excretion of fluorescein

To visualize the fluorescein excretion, 3 µg/ml of fluorescein diacetate (Molecular Probes, Eugene, Oregon) in culture medium was incubated with the cultures at 37 ^{o}C for 45 mins [140]. The cultures were then rinsed and fixed before observing under a

confocal microscope (Fluoview 300, Olympus) using a 40x water lens.

Imaging Process Software (Imaging Process Probe) was used to process the pictures and quantify the fluorescein localization in the inter-cellular sacs (Red color) between hepatocytes (outline in green). The biliary excretory function was indicated by the ratio of the area of fluorescein in intra-cellular sacs to the total area covered by cells.

2.10 Immunofluorescence microscopy

F-actin staining: the cells were fixed using 3.7% paraformaldehyde, blocked in 10% fetal calf serum (FCS) at room temperature for 1 h, permeabilized for 5mins in 0.1% Trion X-100 plus 1% bovine serum albumin (BSA), incubated with TRITC-phalloidin (1μg/ml) for 20mins and then washed three times before imaging.

MRP2/CD147 double staining: Primary anti-CD147 mouse monoclonal antibody (mAb) was purchased from Serotec, Inc. (Raleigh, NC). Primary anti-MRP2 rabbit polyclonal antibody (pAb) was purchased from Sigma-Aldrich. 3.7% paraformaldehyde-fixed samples were blocked in 10% fetal calf serum (FCS) at room temperature for 1 h. Samples were incubated with the primary antibodies (1:10) overnight at 4 ^{o}C, before being rinsed with 1xPBS thrice, each lasting 5mins. Samples were then incubated with the secondary antibodies at room temperature for 1 h and rinsed with 1xPBS before being mounted with FluorSaveTM (Calbiochem, San Diego, CA). The samples were viewed with a confocal microscope (Fluoview 300, Olympus) using 60x water lens.

2.11 Scanning electron microscopy

3.7% paraformaldehyde-fixed samples were rinsed in 1xPBS and then post-fixed with osmium tetraoxide for 1 hour. Dehydration was accomplished using a graded series of

ethanol (25%, 50%, 75%, 95%, and 100%). The samples were then dried for 2 h in absolute alcohol, mounted onto a brass stub and sputter-coated with platinum (JFC-1600, JEOL), before being viewed under a field emission scanning electron microscope (JSM-7400F, JEOL).

2.12 Hepatocytes functional assays

All functional data were normalized to 106 cells. Rat Albumin ELISA Quantitation Kit (Bethyl Laboratories Inc., Montgomery, Texas) was used for the measurement of daily albumin production; Hepatocytes were incubated in culture medium with 2 mM NH4Cl for 90 mins and Urea Nitrogen Kit (Stanbio Laboratory, Boerne, Texas) was used to measure the urea production; The 7-ethoxyresorufin-O-deethylation (EROD) assay was used to measure the deethylation activity of cytochrome P450 (CYP) 1A-associated monooxygenase enzymes, which is initiated by incubating the cultures with 39.2 μM 7-ethoxyresorufin in culture medium at 37 ºC for 4 hours. The amount of resorufin converted by the enzymes was calculated by measuring the resorufin fluorescence in the incubation medium at 543 nm excitation/570 nm emission against resorufin standards using the microplate reader (Tecan Trading AG, Switzerland).

2.13 Statistical analysis

Statistical calculations were carried out by function of T-Test in Microsoft Excel. All values are presented as the mean $\pm$standard deviation unless otherwise noted. Probability values $P<0.05$ were considered significant.

Chapter 3 Enhancing mass transfer efficacy in conventional sandwich configuration by manipulating flow environment

3.1 Limited mass transfer in conventional sandwich culture

Conventional hepatocytes sandwich culture involves culturing cells between two layers of natural extra-cellular matrix support on solid surfaces [129]. The cells are generally seeded onto a collagen-coated polystyrene or glass surfaces and sandwiched by another layer of the collagen on porous membranes so as to allow nutrient access from the culture medium above the sandwich assembly [130]. Sandwiching cells between two layers of extra-cellular matrix support creates an intra-sandwich environment which differs from the extra-sandwich environment defined by culture medium. However, the two layers of extra-cellular matrix support on solid surfaces or membrane can act as mass transfer barriers between the intra-sandwich and extra-sandwich environments causing metabolite accumulation and limited nutrient access in the intra-sandwich environment which in turn influences cellular behaviors. For example, when albumin, the most abundant molecules with a relatively lower diffusion coefficient, a good model molecule to indicate the mass transport properties of metabolites in culture, was examined, it was found that the albumin level in the intra-sandwich environment increased steadily over a period of 24h in static culture (Figure 3.1).

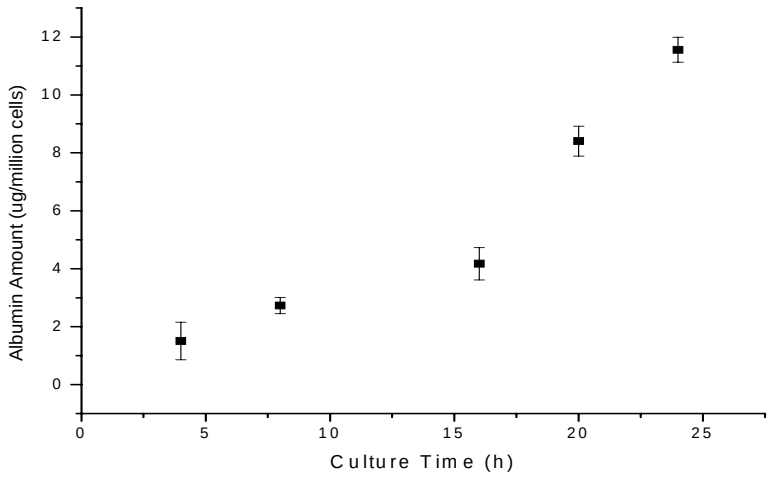


Fig 3.1 Dynamic albumin accumulation in intra-sandwich environment in static hepatocytes sandwich culture.

Previous studies reported that free diffusion coefficients of albumin were in the order of 10^{-11} $m^2 \cdot S^{-1}$ [97, 131]. In gels, diffusion coefficient of albumin is much lower than in free solution, due to the hydrodynamic effects and steric hindrances. The diffusion coefficients of albumin through the collagen membrane and other gel matrix have been observed, ranging from 10^{-15} to 10^{-12} $m^2 \cdot S^{-1}$ [98, 99]. Considering albumin is one of the most abundant molecules produced by hepatocytes *in vitro*, it is reasonable to speculate that albumin also accumulates in intra-sandwich environment of the sandwich construct.

3.2 Effect of mass transfer efficacy on hepatocytes' functions

To investigate whether mass transfer limitation indicated by albumin accumulation in intra-sandwich environment influences hepatocytes functions, we examined how the membrane permeability on the upper side of the sandwich assembly would influence

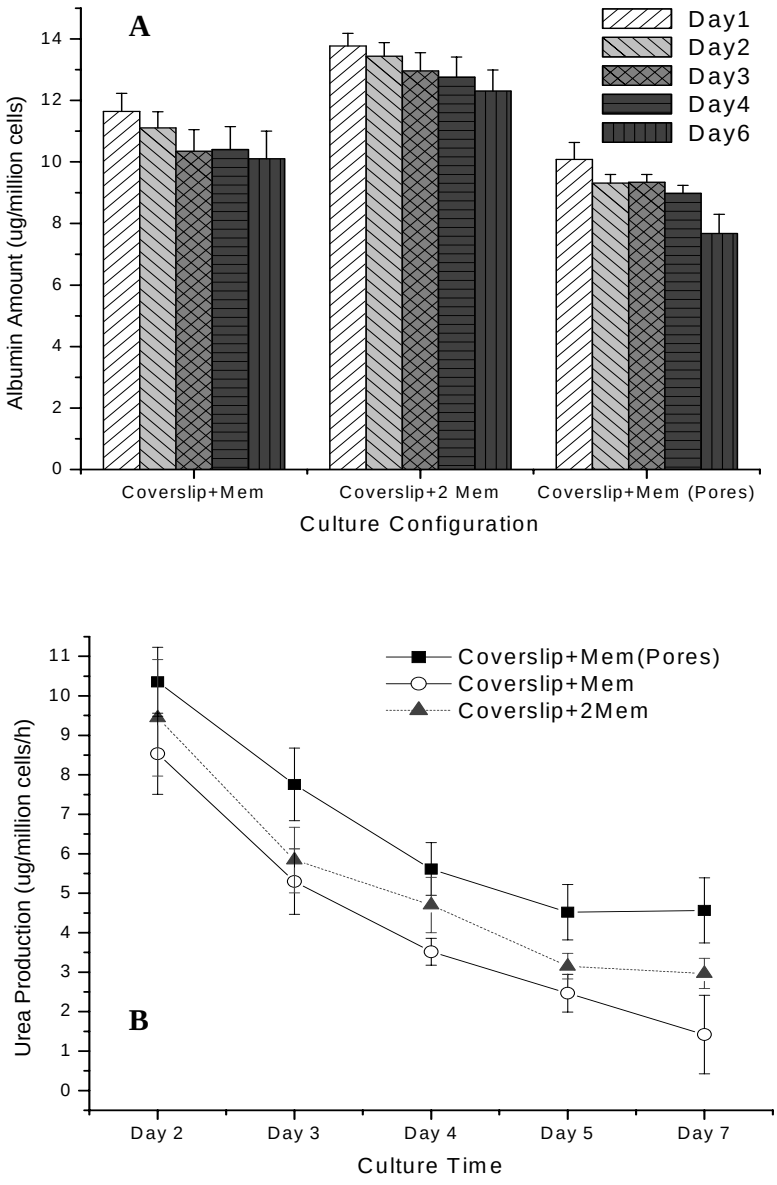


Fig 3.2 Effect of different sandwich culture configurations on the intra-sandwich albumin environment (A), and hence their effect on the urea production (B) at different culture days.

the intra-sandwich albumin accumulation and its corresponding effects on hepatocytes functions. When the upper extra-cellular matrix support consists of two membranes, the albumin accumulation in the intra-sandwich environment is ~ 13.05μg/million cells in average in 6 days of culture (Figure 3.2A). When the upper extra-cellular matrix support consists of one membrane as in our standard sandwich culture, the albumin accumulation in the intra-sandwich environment drops to ~10.70μg/million cells in average. When the upper extra-cellular matrix support consists of a membrane punctured with the tip of 31G needle to increase permeability, the albumin accumulation in intra-sandwich environment drops to ~9.05μg/million cells in average. Hepatocytes function as measured by urea production increases as the intra-sandwich albumin accumulation decreases (Figure 3.2B). It is clear that mass transfer efficacy affect functional maintenance of hepatocytes in sandwich configuration.

3.3 Regulation of mass transfer efficacy by varying perfusion flow rates

As a positive effect of enhanced mass transfer efficacy is demonstrated above, we tried to further improve mass transfer efficacy in a perfusion sandwich culture by flowing culture medium at different flow rates above the upper extra-cellular matrix support on porous membrane in a flat plate sandwich perfusion culture bioreactor. We investigated how different flow rates can influence the intra-sandwich albumin efflux from the intra-sandwich environment to the culture medium (Figure 3.3). We observed that relatively low flow rates of 0.1ml/min to 1ml/min resulted in a diffusion

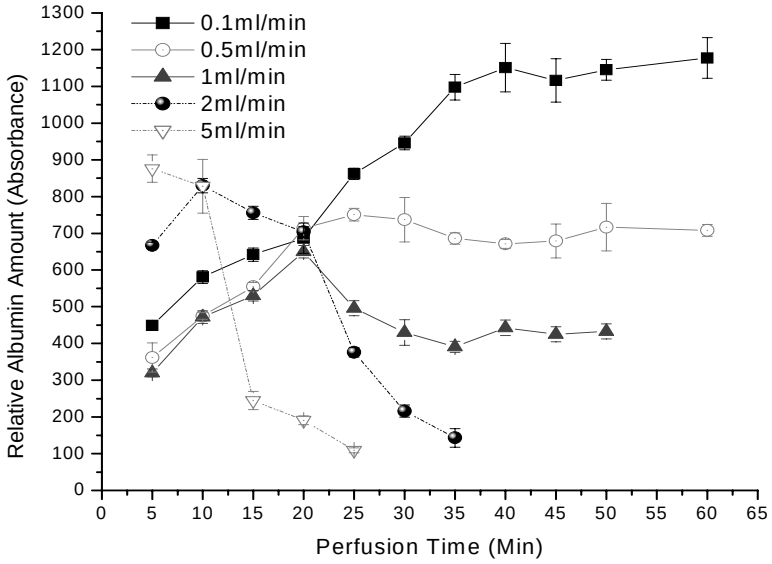


Fig 3.3 The metabolites transport process across the top collagen coated membrane at different flow rates was simulated by a donor-receptor environment model using FITC-BSA at different flow rates in a flat-bed perfusion sandwich bioreactor

dominated transport process of albumin across the upper membrane of the sandwich assembly while flow rates of 2ml/min and 5ml/min led to a convection-dominated transport process. Under relatively low flow rates (0.1ml/min-1ml/min), the diffusion-dominated transport process of albumin across the upper layer of the sandwich construct indicated limited enhancement ability in mass transfer efficacy. While under high flow rates of 2ml/min-5ml/min, all the intra-sandwich albumin are rapidly washed out by a convection-dominated transport process but adversely affected hepatocytes functions. And there still have no control over the different extent of mass transfer efficacy wanted. When the hepatocytes functions as measured by albumin secretion and urea production were examined under different flow rates, hepatocytes maintained higher level of functions in the diffusion-dominated process than

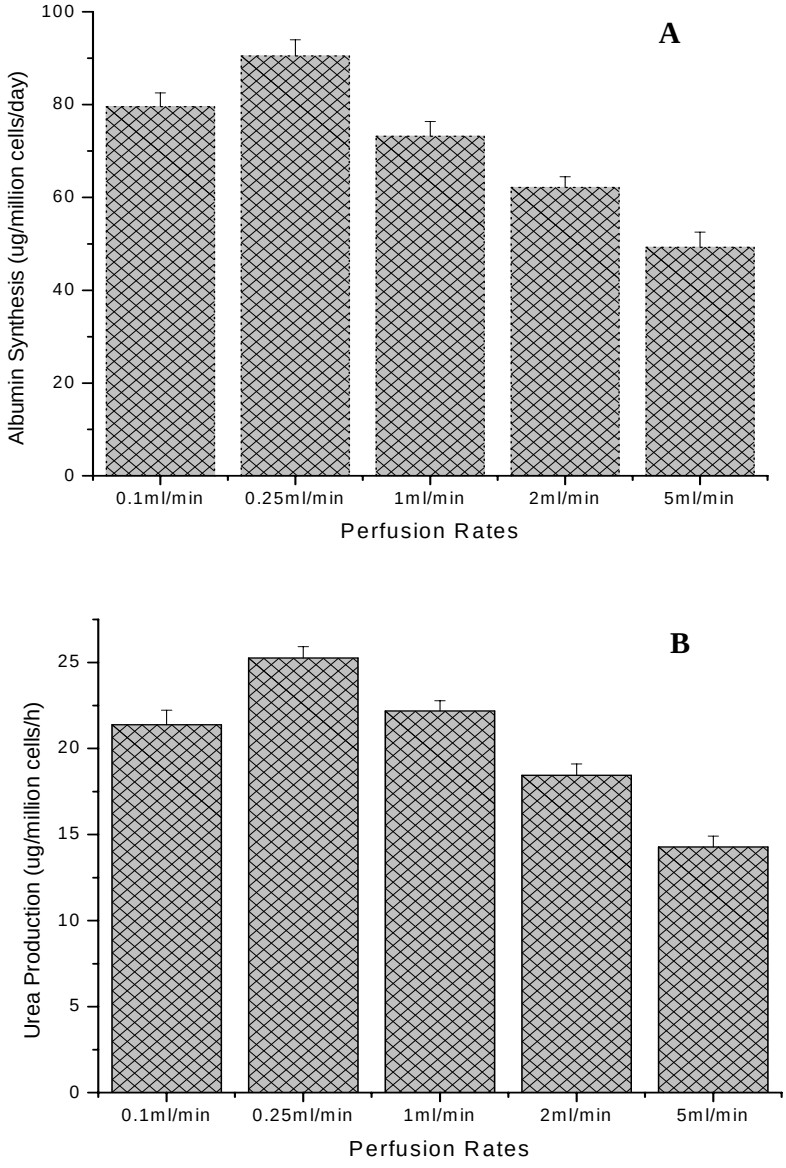


Fig 3.4 Effect of different flow rates in flat-plate bioreactor for sandwich culture on the hepatocytes functions, indicated by Albumin production (A) and Urea synthesis (B) after four day of culture.

in the convection dominated process (Figure 3.4), likely due to the adverse effects that convective flow conditions have imposed onto hepatocytes. This is not surprising since hepatocytes in the *in vivo* liver plate are only exposed to plasma without direct contact with blood, allowing diffusion exchange of plasma proteins, nutrients, and metabolites through sinusoidal cells to realize its myriad functions. Shear stress can damage the cells under high flow conditions and excessive mass exchange can excessively remove good metabolites such as hepatocytes growth factors, a number of hormones, and triiodothyronines (T3) that might be essential for the maintenance of hepatocytes functions [132].

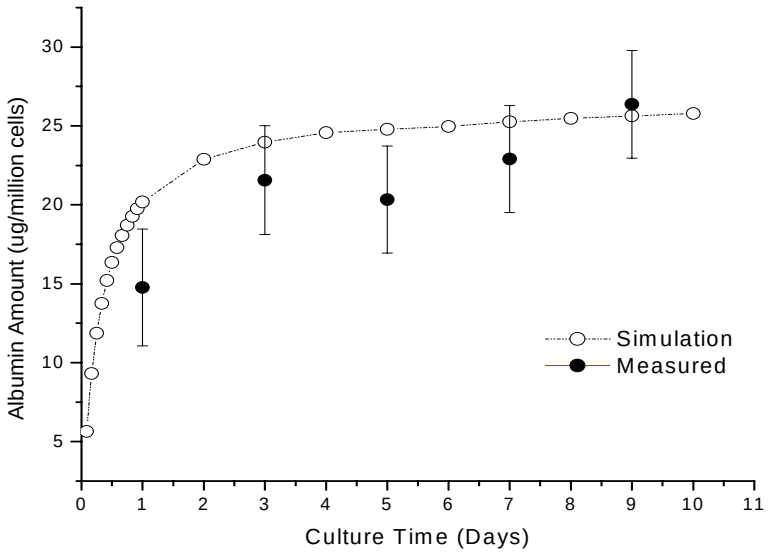


Fig 3.5 The albumin level in intra-sandwich environment under flow rate of 0.25ml/min with the simulation based on the permeability coefficients at different culture period (D).

Unfortunately, the intra-sandwich albumin accumulates significantly under the low flow rate (0.25ml/min) although it yields the optimal hepatocytes functions (Figure

3.5). The simulation results based on proposed diffusion model and permeability coefficients measured (2.95×10^{-8} cm^2/s) at different flow rates also predict the similar intra-sandwich albumin accumulation. It is difficult to control the mass transfer efficacy across ECM layers tremendously by only varying perfusion flow rates in the range of diffusion dominated flow rates. The limited mass transfer ability indicated by intra-sandwich accumulation of albumin will still impact the hepatocytes functions adversely, if not effectively regulated. It is also impossible to control mass transfer efficacy in convection dominated flow rates, as all the albumin was washed out immediately and there still have no control over it.

3.4 Regulation of mass transfer efficacy by a separate drainage

To effectively regulate mass transfer efficacy as indicated by intra-sandwich albumin accumulation independently from varying perfusion flow rates, we have developed a novel bioreactor with a separate drainage channel directly connected to the intra-sandwich environment. Two flow paths were established in this system: one is the flow path above the upper layer of the extra-cellular matrix on porous membrane with the flow rate of 0.25ml/min that yields the optimal hepatocytes functions. Another is a branched flow path from the first flow path through intra-sandwich environment and the side pores of the sandwich carrier into the extra-carrier space in the bioreactor chamber such that the intra-sandwich environment could be regulated directly through controlling the flow rates in this drainage flow path. The effects of different drainage rate on intra-sandwich albumin accumulation and its subsequent effect on hepatocytes

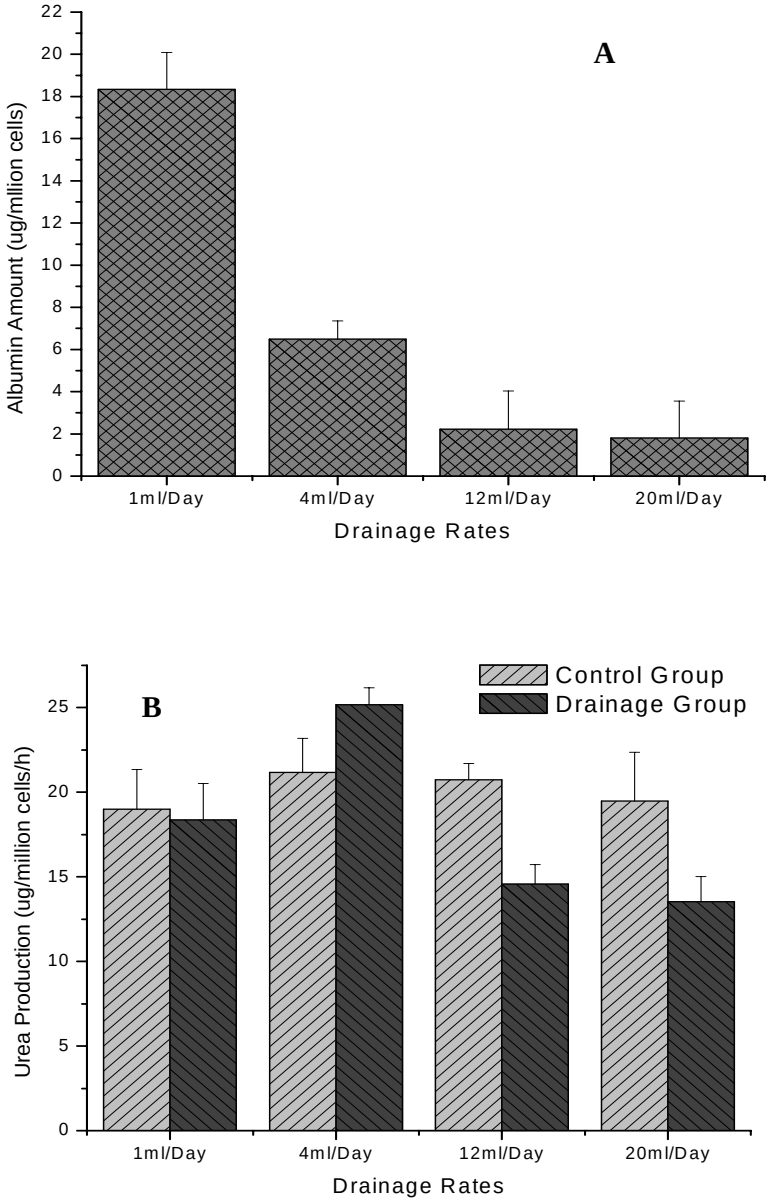


Fig. 3.6 Effect of different drainage rates on the albumin level in intra-sandwich environment (A) and on the urea production (B) after four day of culture.

were investigated (Figure 3.6). The intra-sandwich albumin accumulation was reduced from 18.35μg/million cells (drainage rate of 1ml/day) to 6.50μg/million cells (drainage rate of 4ml/day), 2.22μg/million cells (drainage rate of 12ml/day) and 1.80μg/million cells (drainage rate of 20ml/day) after 4 days of perfusion culture. With drainage rate of 4ml/day, urea production by hepatocytes is 20% higher than the no-drainage control after 4 days of perfusion culture; with drainage rate of 1ml/day, no obvious difference in urea production was observed between the drainage group and the no-drainage control. With drainage rates of 12ml/day and 20ml/day, the urea production were ~30% lower than the no-drainage control. Therefore, the mass transfer efficacy can be effectively regulated and there is an optimal drainage rate of ~4ml/day that yields the best urea production after 4 days of perfusion culture. The intra-sandwich albumin accumulation at the end of the 4-day perfusion culture is reduced ~74% from the no-drainage control.

3.5 Maintenance of hepatocytes functions in perfusion sandwich culture with separate drainage

We further characterized the hepatocytes functions in perfusion sandwich culture with optimized mass transfer efficacy (the flow rate of 0.25ml/min and drainage rate of 4ml/day). Hepatocytes functions as measured by urea production and albumin secretion were better maintained for two weeks with up to ~15% higher levels in the perfusion sandwich culture with direct drainage than the no-drainage control. There is

also a significantly less drop in hepatocytes functions in the perfusion sandwich culture with direct drainage over the two week period than the no-drainage control (Figure 3.7).

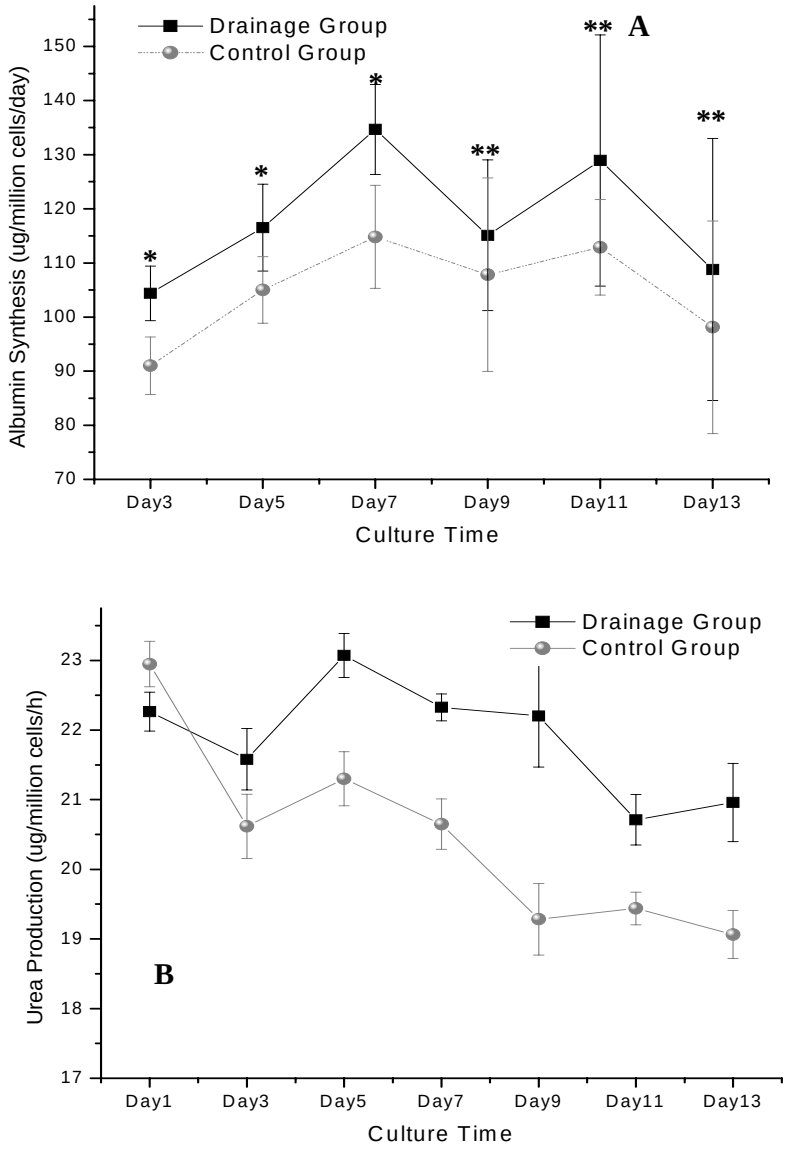


Fig. 3.7 Hepatocytes functions shown by albumin production (A) and urea production (B) in culture period of two weeks under perfusion culture with optimized drainage rate. [*Student's T-test ($P<0.05$) were performed between drainage and control groups; ** not significant]

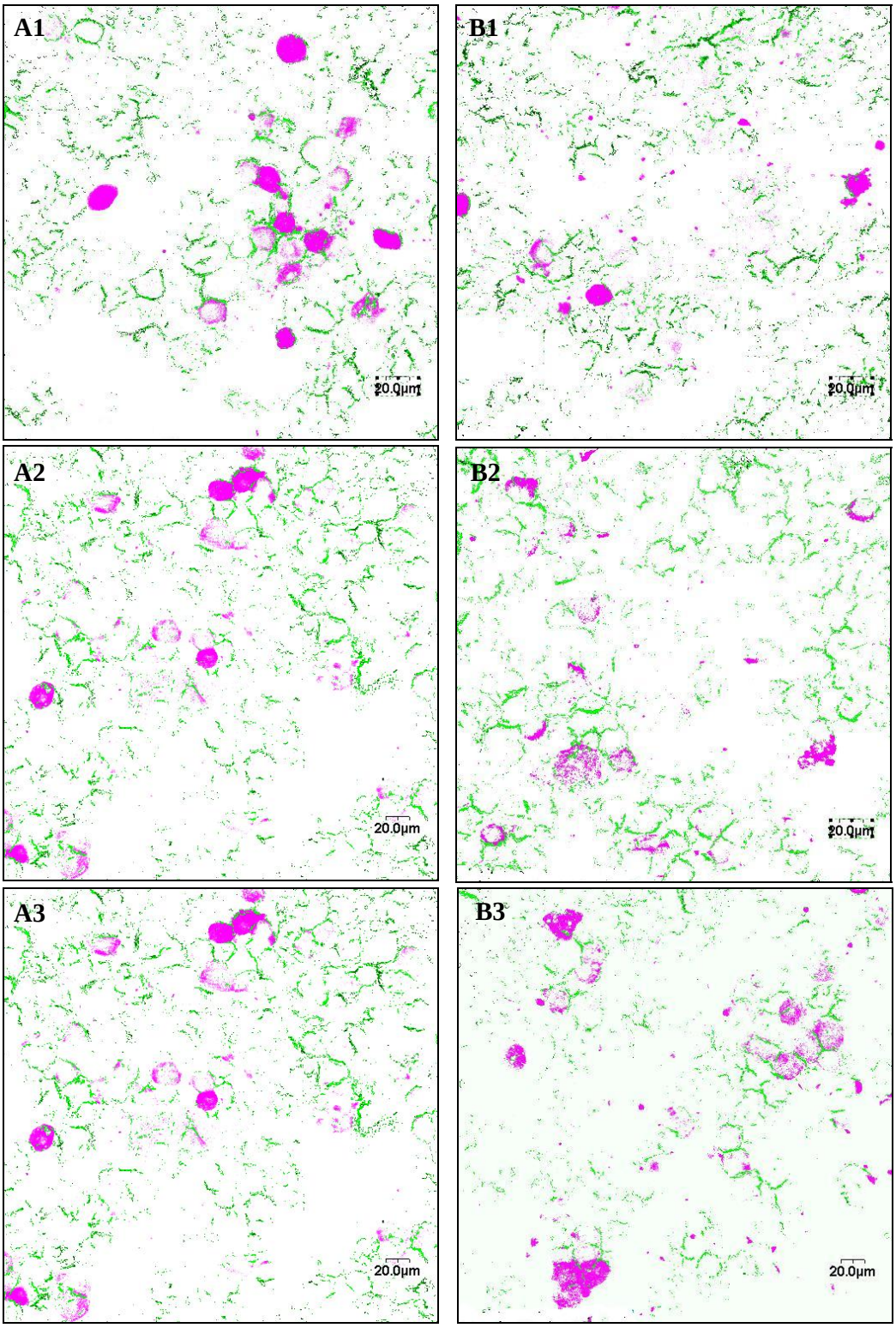


Fig 3.8 Excretory function of hepatocytes indicated by FDA staining in the optimized drainage culture condition (B) after 2 (B1) 6 (B2) and 12 (B3) day of culture compared with control group which do not incorporate drainage (A1, A2and A3).

To test the functional structure features in the separate drainage bioreactor, excretory function of hepatocytes was evaluated by imaging and quantifying the fluorescein localization in the inter-cellular sacs between hepatocytes. The fluorescein in the inter-cellular sacs is derived from fluorescein diacetate (FDA) being metabolized and excreted by hepatocytes [133]. As is shown in Figure 3.8, more fluorescein (red color) was concentrated in the sacs between hepatocytes (outline in green) from 48 h to at least 12 days of perfusion culture with direct drainage than the no-drainage control. This is further supported by quantifying the ratio of the area of fluorescein to the total area covered by cells (Figure 3.9). Therefore, hepatocytes in perfusion sandwich culture with direct drainage of up to ~74% of the intra-sandwich albumin can maintain a higher level of cellular functions and functional polarity than the no-drainage control for extended period of time.

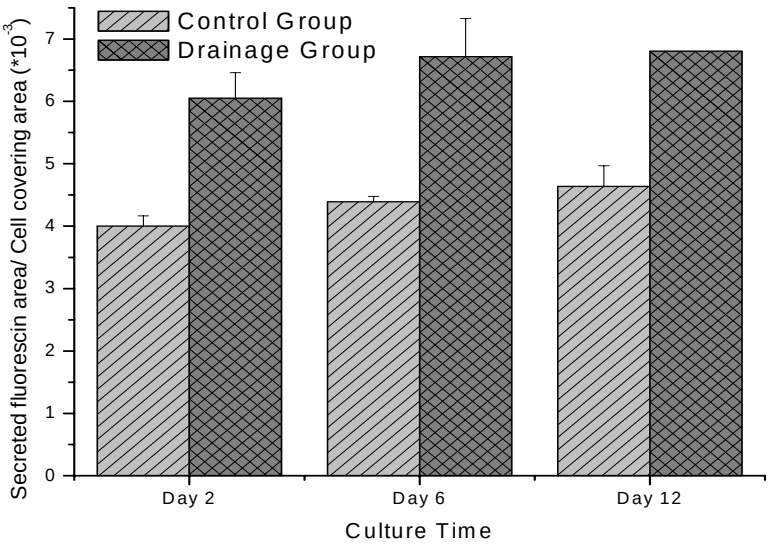


Fig 3.9 The excretory function quantified by the ratio of the area of fluorescein in intra-cellular sacs to the total area covered by cells

Therefore, the regulation of mass transfer efficacy in intra-sandwich environment can be somehow achieved in the novel separate drainage bioreactors and a higher level of cellular functions and functional polarity were observed with optimized mass transfer efficacy.

Chapter 4 Engineering Novel Synthetic Sandwich Configuration with High Mass Transfer Efficacy

We know that in conventional sandwich configuration, the inherited mass transfer barriers are mainly caused by the introduction of natural ECMs such as collagen, which slow down the exchange of nutrients, products, and chemical signals with the bulk of the medium. We tried to overcome mass transfer barriers in sandwich culture by using precisely-controlled synthetic polymer scaffold with proper functional modification to replace the natural ECMs. To develop a novel synthetic sandwich configuration which has higher mass transfer efficacy, while maintains high differentiated functions and polarity as what is observed in conventional sandwich configuration. This novel approach was inspired by our finding of a special 3D hepatocytes stage on galactose-conjugated PET film.

4.1 Galactose-conjugated PET film as the bottom support of sandwich configuration

4.1.1 Fabrication and characterization of PET film with Gal-ligands

Acrylic acid was grafted onto the poly (ethylene terephthalate) (PET) film by argon-plasma treatment and UV-induced copolymerization. The effectiveness of the grafting was demonstrated by XPS analysis (Figure 4.1). The XPS wide scan spectrum of the pristine PET film showed peaks corresponding to C 1s (binding energy, 285eV) and O 1s (binding energy, 532eV), which revealed the presence of carbon and oxygen signals.

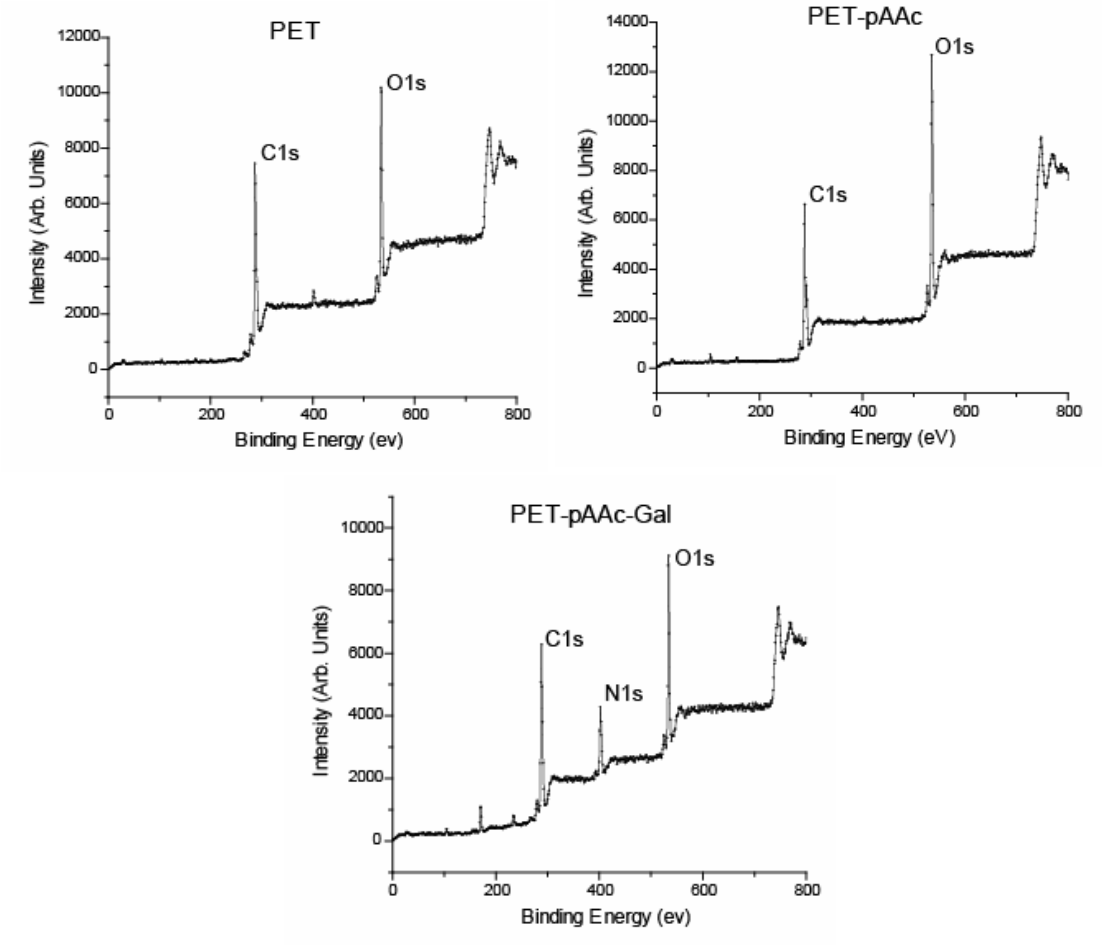


Fig 4.1 XPS wide scanning spectrums of PET(1), PET-g-AAc(2), PET-gal (3) which showed the successful grafting of acrylic acids and following immobilization of Gal ligands onto the PET film.

The spectrum of PET-pAAc film showed the same peaks as pristine PET film; however, the relative intensity ratio of oxygen to carbon peaks is higher in PET-pAAC film than in pristine PET film. The pAAc grafting density was quantified by TBO colorimetric assay. PET-pAAc substrata with carboxyl group densities from 8.2±2.3 to 258.2±24.2nmol/cm^2 could be obtained by varying the initial concentration of the acrylic acid monomer solution from 1%-5%. As reported previously, the difference in

density of carboxylic groups and conjugated galactose ligands was not expected to lead to significant differences in hepatocytes 3D spheroids formation and functional maintenance when the densities went above certain value. 3.75% acrylic acid monomer solution was chosen to fabricate PET-pAAc with carboxyl group density of 78.5±10.2 nmol/cm^2 for the following ligands conjugation and cell culture work in order to achieve reasonably high ligand-conjugation density using the relatively inefficient two-step 'EDC chemistry'.

Gal ligand (AHG) were conjugated onto the PET-pAAc film, and successful conjugation of ligands was confirmed by XPS (Figure 4.1). In contrast to pristine PET and PET-pAAc, a new peak corresponding to N 1s (binding energy, 400eV) appeared in the spectra of the PET-Gal. To measure the amount of AHG conjugated onto the films, we removed the conjugated AHG from the film by acid hydrolysis, and quantify the hydrolyzed AHG by RP-HPLC with fluorescence detector after derivatizing the α-amine on the hydrolyzed AHG to fluorescent substances. The final density of AHG of PET-Gal is 5.92±0.74nmol/cm^2.

4.1.2 Dynamic process of hepatocytes self-assembly on galactosylated substratum

Primary rat hepatocytes were seeded onto the 35-mm galactose-bottom Willco dish at a density of 10^5 cells/cm^2 for live-imaging of spheroid formation. During hepatocytes self-assembly, dramatic changes occur in cell morphology and substratum coverage as shown by confocal transmission images (Figure 4.2) and SEM images (Figure 4.3). Hepatocytes on galactosylated substratum undergo 2 cycles of cell-aggregation to form mature spheroids. Single hepatocytes seeded on this galactosylated substratum form small aggregation of hepatocytes within 12 h with reduced area of coverage of

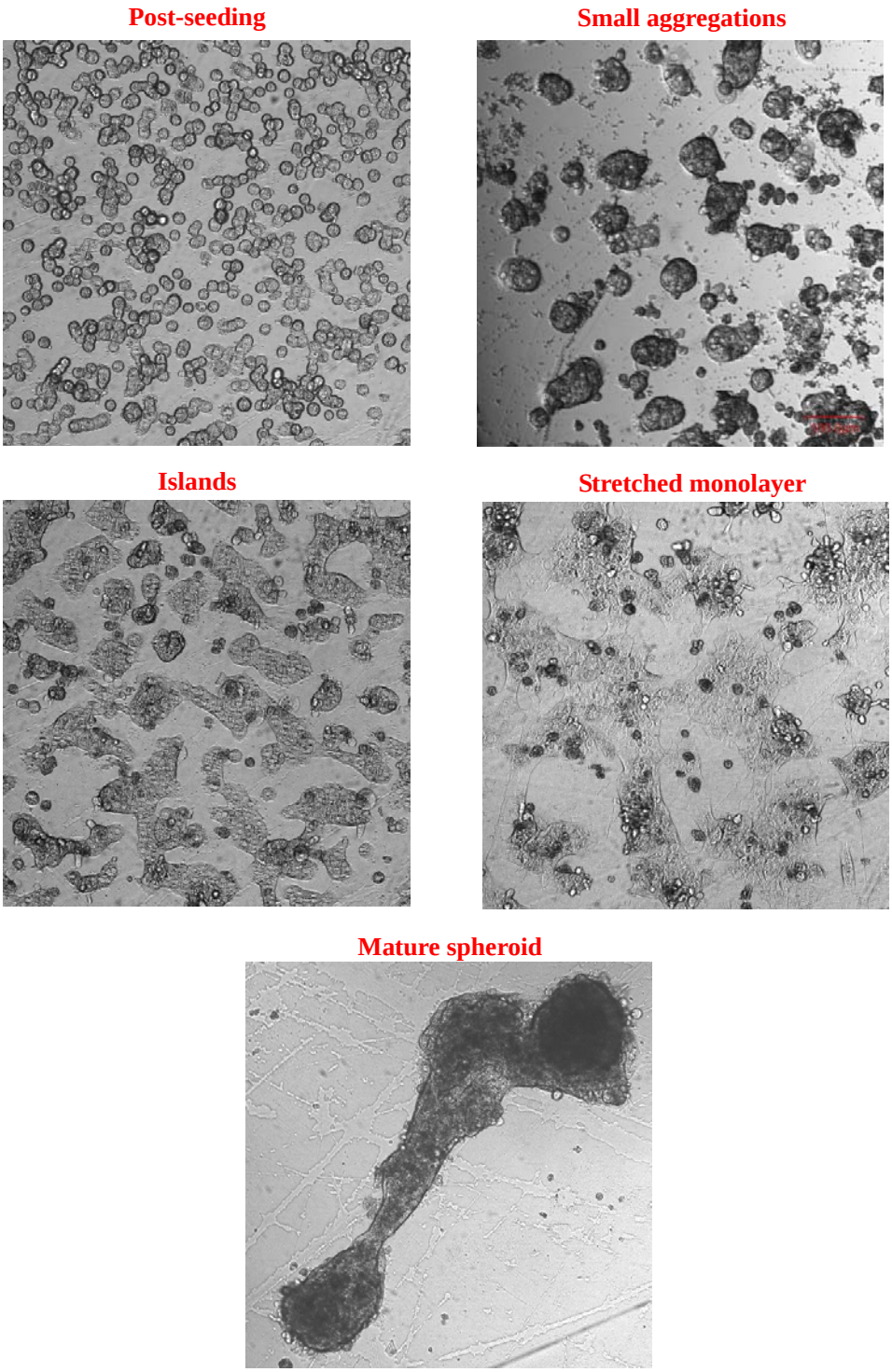


Fig 4.2 Dynamic morphogenesis of hepatocytes' self assembly on Gal-PET film using the confocal transmission imaging

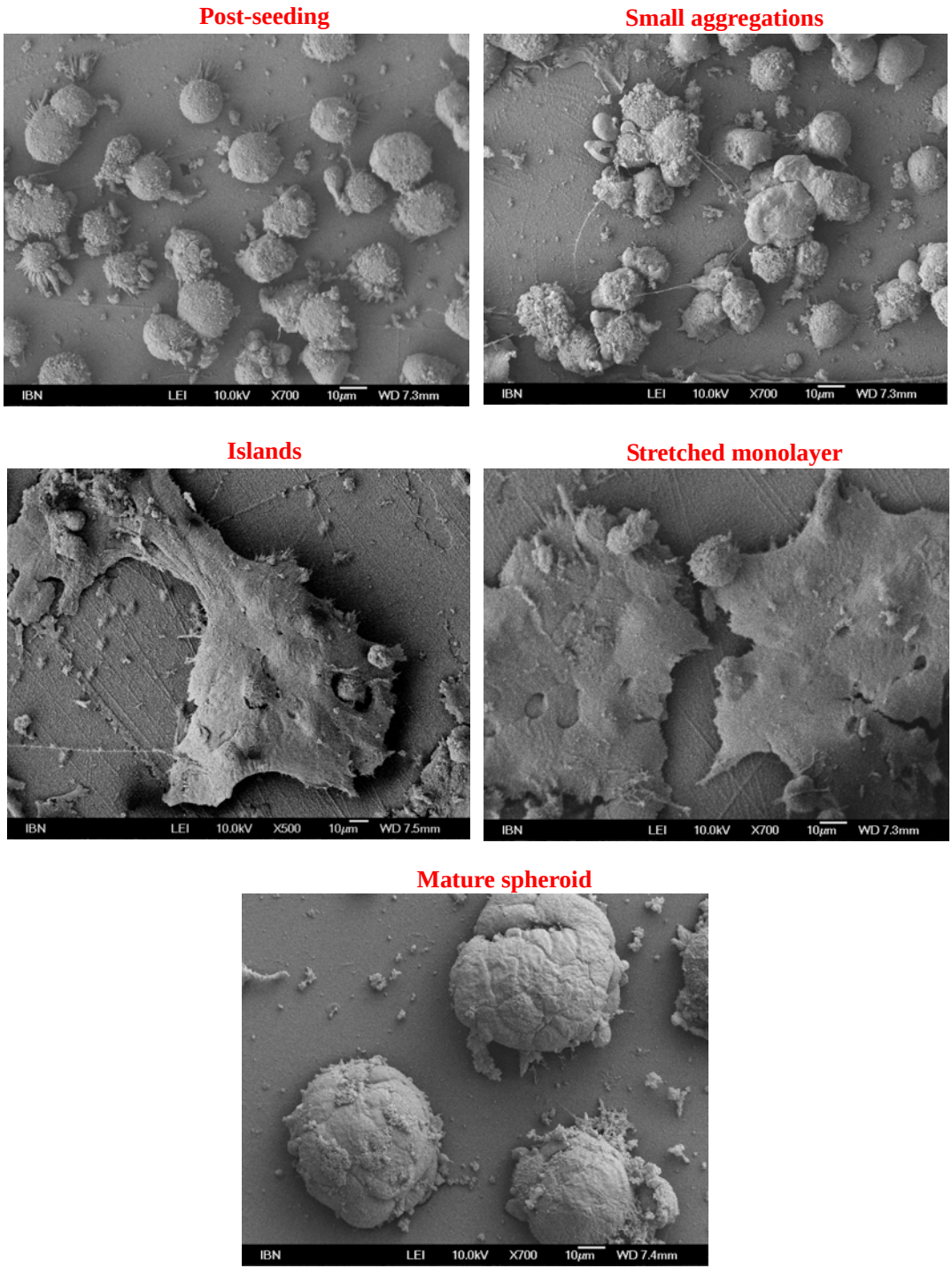


Fig 4.3 Dynamic morphogenesis of hepatocytes' self assembly on PET film using SEM at different stages

the substratum. Migration of the cells facilitates the establishment of cell-cell contacts and contractions among small aggregates which gradually merged into larger 'island-like' clusters after one day. The island-like clusters undergo further spreading to form monolayer within two days with the maximum substratum coverage. Due to the strong cell-cell contraction, the cells at the edge of the pre-spheroid monolayer was stretched to fold into multilayer and finally compacted into mature and larger 3D spheroids after three days which will finally detached from the substratum. The total process exhibited five different stages with two cycle of spheroid formation.

4.1.3 3D monolayer stages in galactosylated PET film

To evaluate the application potential of this novel pre-spheroid 3D monolayer configuration, we compared the liver-specific functions such as synthetic, detoxifying and metabolic activities of hepatocytes in this transient stage cultured at Day 2 with conventional 2D monolayer at Day 2 as well as 3D spheroids at Day 3. Figure 4.4 shows that the albumin secretion and 7-ethoxyresorufin-O-deethylation cytochrome P450 activity induced by 3MC of hepatocytes in pre-spheroids 3D monolayer are significantly higher than that of the conventional 2D monolayer and comparable to that of 3D spheroids. Urea synthesis doesn't show significant difference among these three configurations. SEM images with higher magnification were used to further show the details of cell morphology and cell-cell interactions among different culture configurations (Figure 4.5). 3D spheroids exhibit smooth surface without distinguishable cell-cell boundaries. Cells within the pre-spheroid 3D monolayer are tightly interacting with each other with protrusions extending from one cell to another, whereas cell-cell interaction in the conventional 2D monolayer were generally more loose with distinguishable cell-cell boundary .

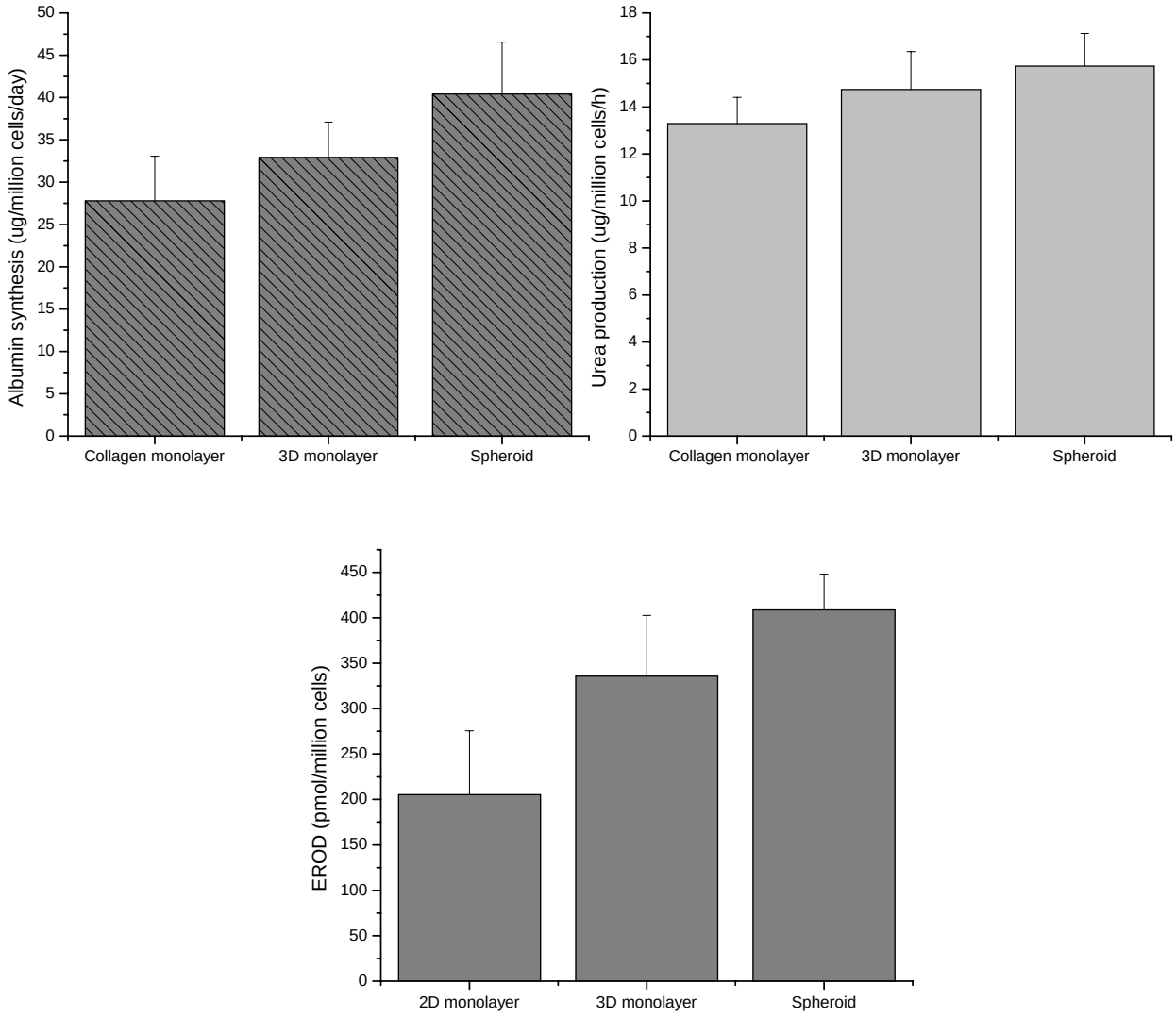


Fig 4.4 Liver specific functions: Albumin synthesis, Urea production and EROD activity under different culture conditions, including 2D monolayer on collagen and 3D monolayer and spheroid.

4.2 Overlaying of 3D monolayer with functionalized PET membrane

4.2.1 Permeability of selected PET membrane

To have enhanced mass transfer efficacy in the synthetic sandwich configuration, high

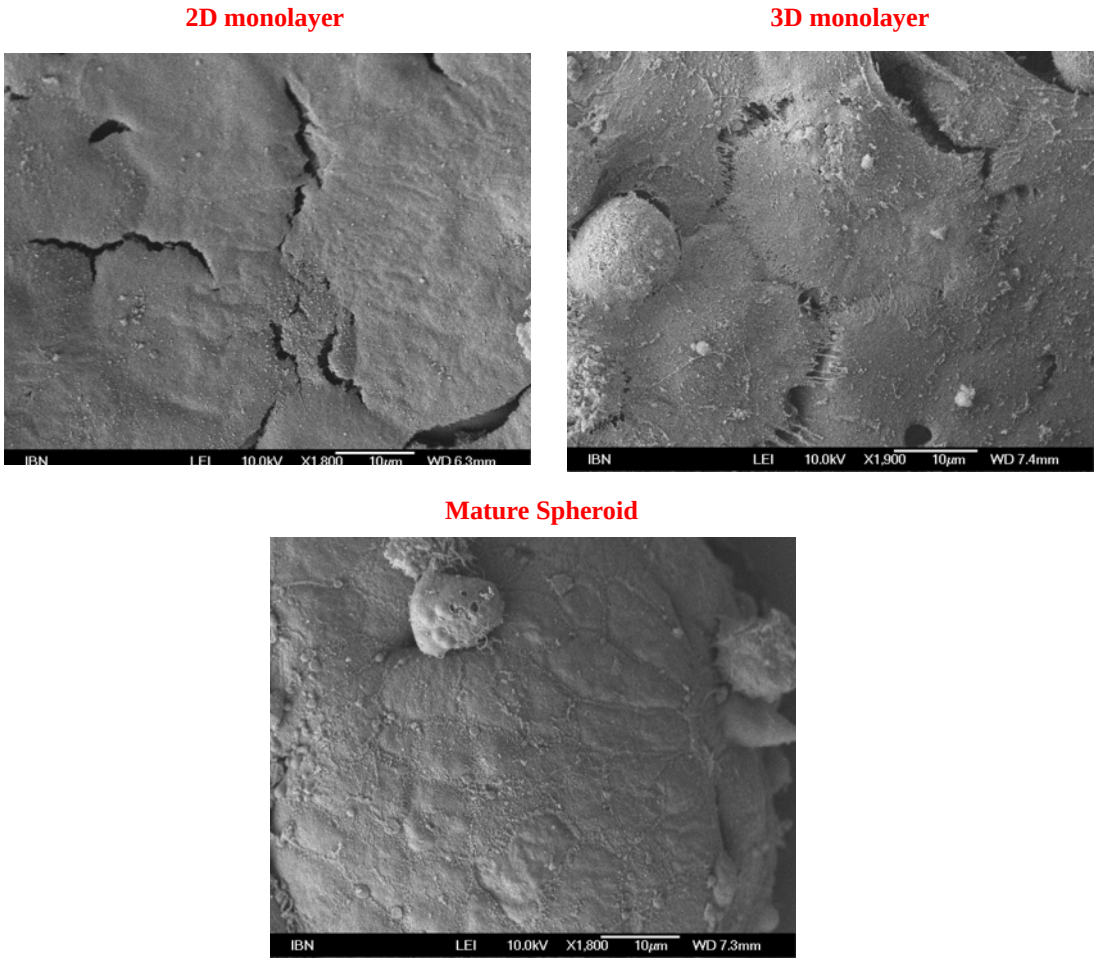


Fig 4.5 SEM pictures of hepatocytes in various culture conditions, including 2D monolayer, 3D monolayer and mature spheroid

permeable PET membrane was selected as the support at the top layer of 3D monolayer. We investigated the mass transfer efficacy across PET membrane and collagen layer using FITC-dextrans of various molecular weights of 9.5, 70, 150 kDa, representative of a range of molecules of different sizes in the culture medium (Table 4.1). Two fold increase of mass transfer efficacy was observed with the PET membranes over the collagen layer normally used in conventional collagen sandwich. The diffusivities of the FITC-dextrans were inversely related to their molecular weights and diffusivity of the order of 10^{-8} cm^2/s was displayed in both conditions,

TABLE 4.1 Diffusivity of dextran of various molecular weights across the modified PET membrane and collagen layer.

Molecular Weights (kDa)	Diffusivity × 10^8 (cm^2/s) [PET]	Diffusivity × 10^8 (cm^2/s) [collagen]
9.5	4.58±0.44	2.26±0.56
70	4.38±0.56	2.04±0.69
150	3.53±0.49	1.70±0.53

Which is comparable to other porous membranes such as poly (ε-caprolactone) or collagen sheets. The results indicated that overlaying 3D monolayer using PET membrane can provide a better mass transfer environment for hepatocytes in sandwich than the conventional collagen sandwich.

4.2.2 Fabrication and characterization of bioactive PET membrane

TBO assay showed high density of carboxylic groups (39.31±1.6 nmol/cm^2) present on the PET track-etched membrane from Whatman which was comparable to the poly-acrylic acid grafted PET film (78.5±10.2 nmol/cm^2). Oxidation by KMnO4 and H2SO4 did not show significant effect to increase the carboxylic group's density (41.32±2.1 nmol/cm2), which indicated that the PET track-etched membrane might have been oxidized by the manufacturer.

RGD peptide (GRGDS) or Gal ligand (AHG) were conjugated onto the PET track-etched membrane activated by EDC and sulfo-NHS. Successful conjugation of ligands was confirmed by XPS (Figure 4.6). To measure the amount of the GRGDS and/or AHG conjugated onto the films, we removed the conjugated GRGDS or AHG

from the PET membrane by acid hydrolysis, and quantified the hydrolyzed GRGDS and AHG by RP-HPLC with fluorescence detector after derivatizing the α-amine on the hydrolyzed GRGDS and AHG to fluorescent substances. The final density of the conjugated GRGDS and AHG on the PET track-etched membrane was 0.63±0.14nmol/cm^2 and 1.48±0.65nmol/cm^2 which showed around 1.5% and 3.5 % surface functionality respectively. In comparison, relatively higher galactose density (5.92±0.74 nmol/cm^2) and surface functionality (7.5%) were achieved in the PET-f-Gal.

4.3 Effect of Various functional overlay on hepatocytes

The synthetic sandwich configurations were formed by overlaying an unstable 3D monolayer with the Gal-, RGD- or non modified PET membrane. We first compared the performance of these three synthetic sandwich configurations in terms of their ability in F-actin localization, functional maintenance and morphology stabilization. F-actin localization in the cell-cell contact regions has been suggested to be a direct indicator of polarity initiation in the conventional collagen sandwich. Overlay of 3D monolayer with Gal- and RGD-PET membrane induced dramatic F-actin changes with the localization of F-actin in cell-cell contact areas after 12h of sandwich culture, while overlay of non-modified PET membrane seemed to have no positive effect on the polarity genesis (Figure 4.7). Functional improvement of sandwich over 3D monolayer was observed in the sandwich configurations with overlay of Gal- and RGD-PET membrane, and the highest urea production and P450 enzyme activity were maintained in the RGD-PET membrane (Figure 4.8). As the 3D monolayer tends to form the spheroid after 2 days of monolayer culture, we tested the ability of stabilization of monolayer stages of these three sandwich configurations. It was found

that 3D monolayer overlaid with Gal- and non modified PET membrane formed the

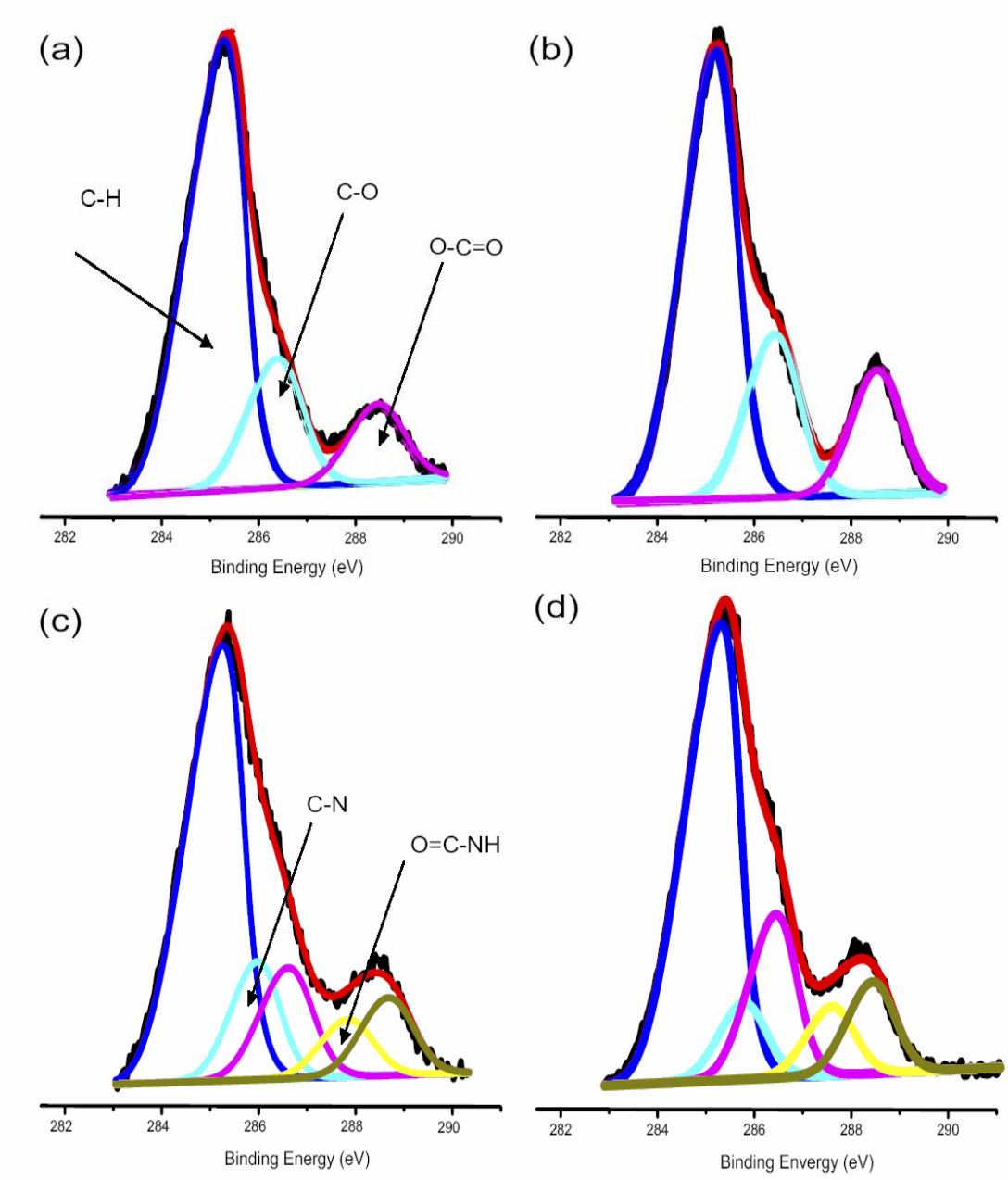


Fig 4.6 XPS C1s core-level spectra of (a) the pristine PET track-etched membrane; (b) the oxidized PET membrane; (c) the RGD conjugated PET membrane and (d) the galactosylated PET membrane.

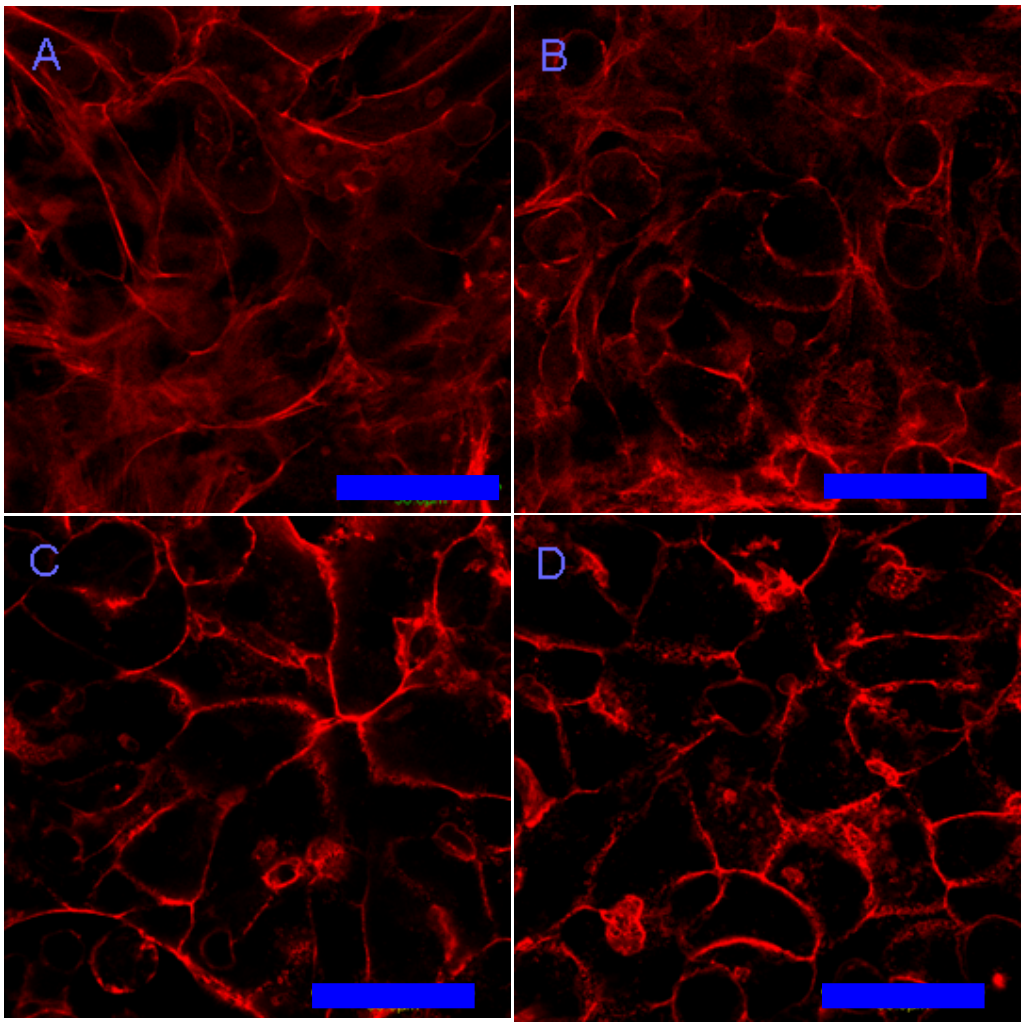


Fig 4.7 Effect of overlay of 3D monolayer with Non-modified PET membrane (B), Gal-PET membrane (C) and RGD-PET membrane (D), compared with no overlay group (A). Scale 50um.

multi-layer structures after one week of culture, while overlay with RGD can stabilize the 3D monolayer for at least one week (Figure 4.9). These results suggests that RGD-PET membrane will be the ideal synthetic functional support for 3D monolayer and the sandwich configuration formed by overlay of RGD-PET membrane (RGD sandwich) have the best performance among the three configurations.

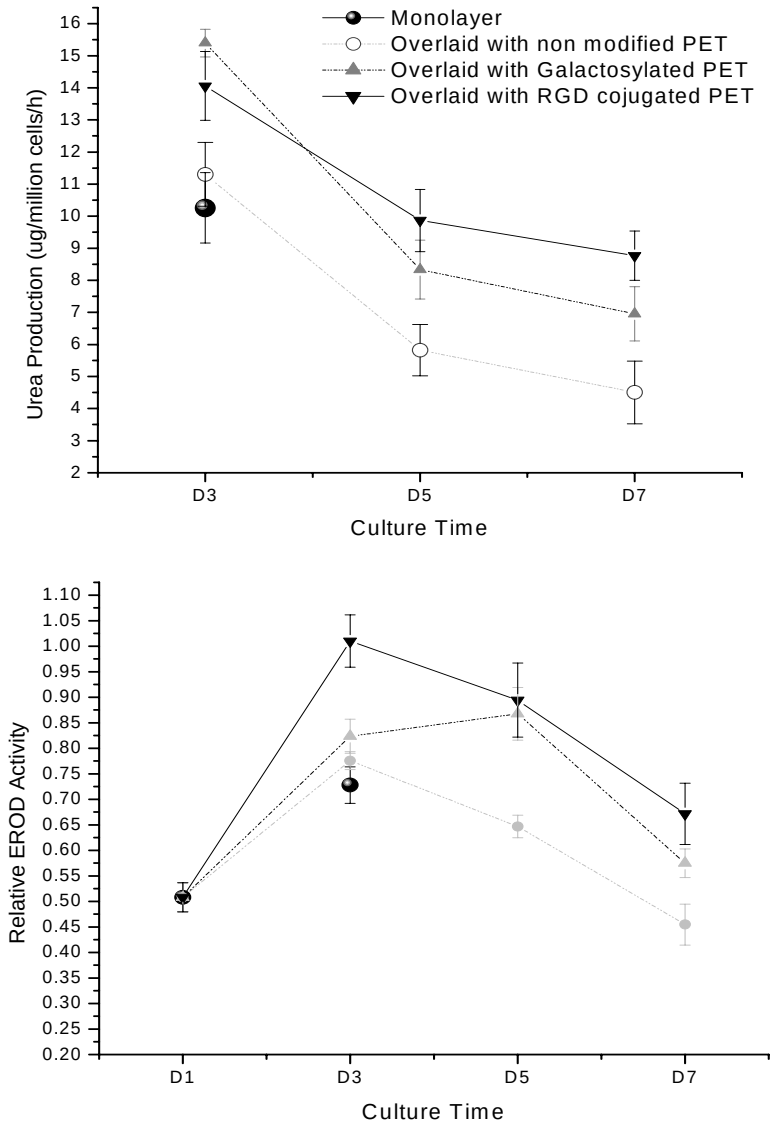


Fig 4.8 Effect of overlay of 3D monolayer with different functionalized PET membrane on hepatocytes' functions.

The synthetic sandwich configuration was formed by the overlay of a novel 3D monolayer formed in galactosylated PET film with Gal-, RGD- or Non-PET

membranes. This unstable 3D monolayer stage before spheroid formation after 1 day

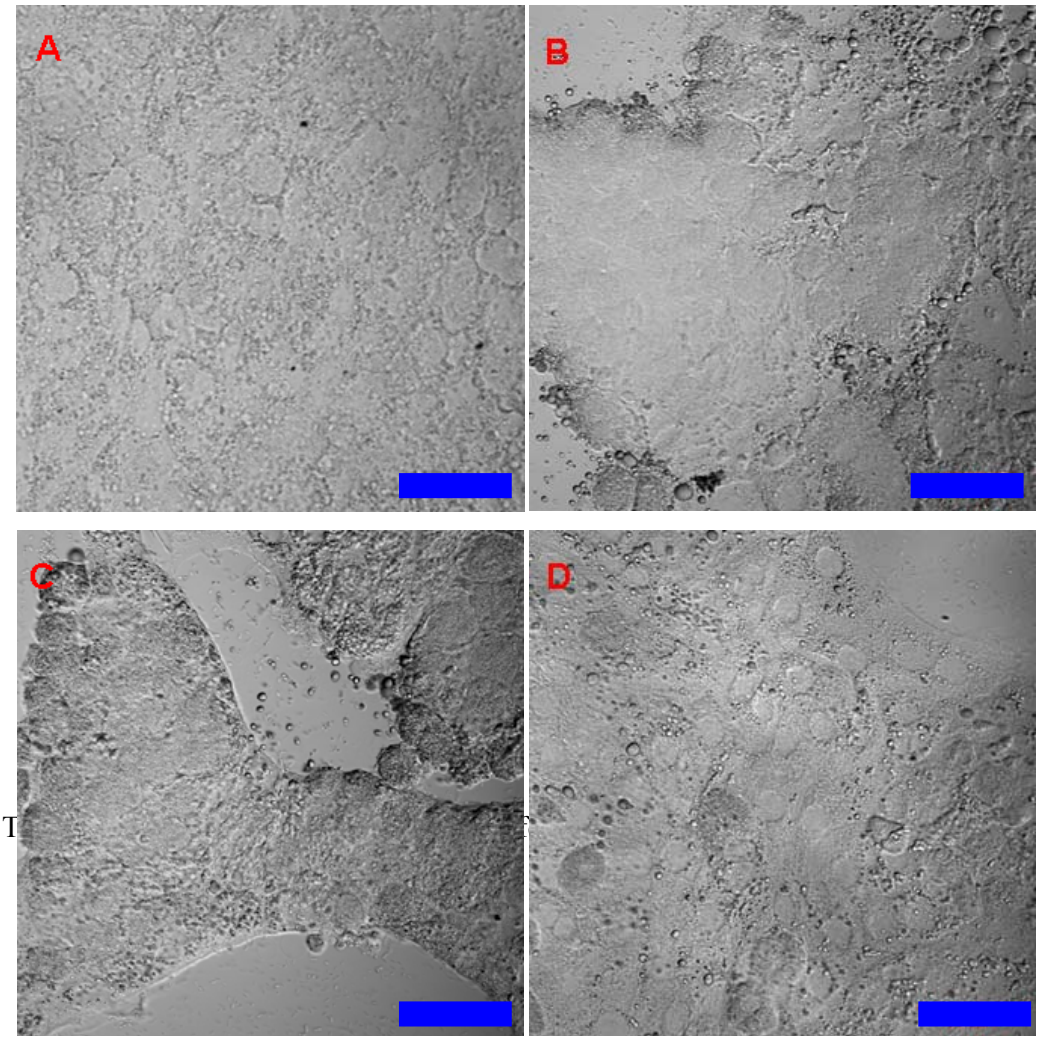


Fig 4.9 Morphology of hepatocytes under the sandwich configurations with Non-modified PET membrane (B), RGD-PET membrane (C) and Gal-PET membrane (D) overlay after one week of culture, compared with no overlay group (A). Scale 50um

of culture on galactosylated substratum exhibited improved cellular structure and polarities, enhanced cell-cell interactions, better differentiated functionalities than hepatocytes monolayer on collagen coated surface.

The overlay of different supports in PET membrane may act as 1): a physical force on the top of cells to prohibit 3D cells from forming spheroid and 2): functional chemical support for the induction of further structural changes and stabilization. As the non-

modified overlay nearly did not induce any effect on 3D monolayer, we could deduce that the functional support played an essential role. In both sandwich configurations with functional supports at the top, hepatocytes were exposed to galactose or RGD ligand at the top and galactose at the bottom. The synergistic interplay between the effects of those two ligand-receptor interactions may be expected. RGD- sandwich configuration showed better performance in terms of function maintenance, polarity formation and morphology stabilization. It is known that the ligand-receptor interaction between galactose and ASGPR is relatively weak and a non-integrin pathway is involved [122]. In contrast, RGD-integrin interactions have been shown to have strong influence to induce downstream signaling pathway to cause the redistribution of the cytoskeleton, formation of focal adhesion complex, enhancement of cell-cell interaction in many studies [127].

In addition, hepatocytes attached to RGD-conjugated substratum keep spreading morphology as monolayer, exhibiting similar characters as monolayer formed in collagen, while hepatocytes in galactosylated substratum tend to form spheroid after several days of culture. In this case, RGD overlay, by the integrin complex, helped to stabilize the 3D monolayer and induced a series of structural changes comparable with what is induced by collagen overlay through the intergrin complex. We observed a similar and comparable polarity formation process in the synthetic sandwich configuration as what was in the conventional collagen sandwich. Less stabilization effect was exposed to the hepatocytes in the sandwich group with Gal-PET membrane at the top.

4.4 Hepatocytes sandwiched between Gal-PET film at the bottom and RGD-PET membrane at the top

4.4.1 Cell-cell interaction

SEM micrographs of hepatocytes after 48h of sandwich culture were used to compare the effects of both sandwich configurations on cell morphology and cell-cell interactions. Hepatocytes cultured in the synthetic sandwich configuration formed a more tightly organized cell-cell interaction with featured cellular protrusions extending from one cell to another cell. In addition, cell boundaries have been merged to each other and cannot be clearly identified; whereas cells in the conventional collagen sandwich were more rounded and generally more loosely interacting with other cells (Figure 4.10). The results show that hepatocytes in synthetic sandwich configuration featured tighter cell-cell interactions over conventional collagen sandwich.

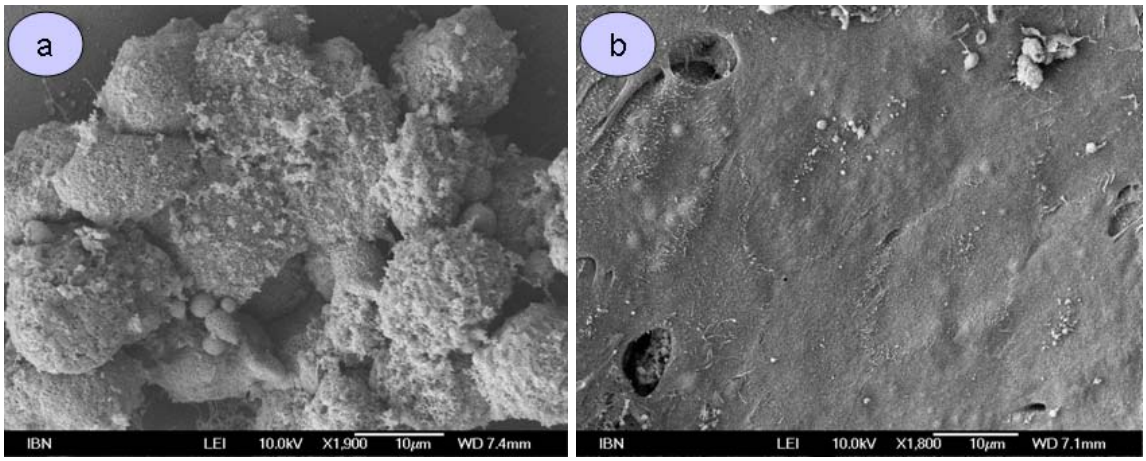


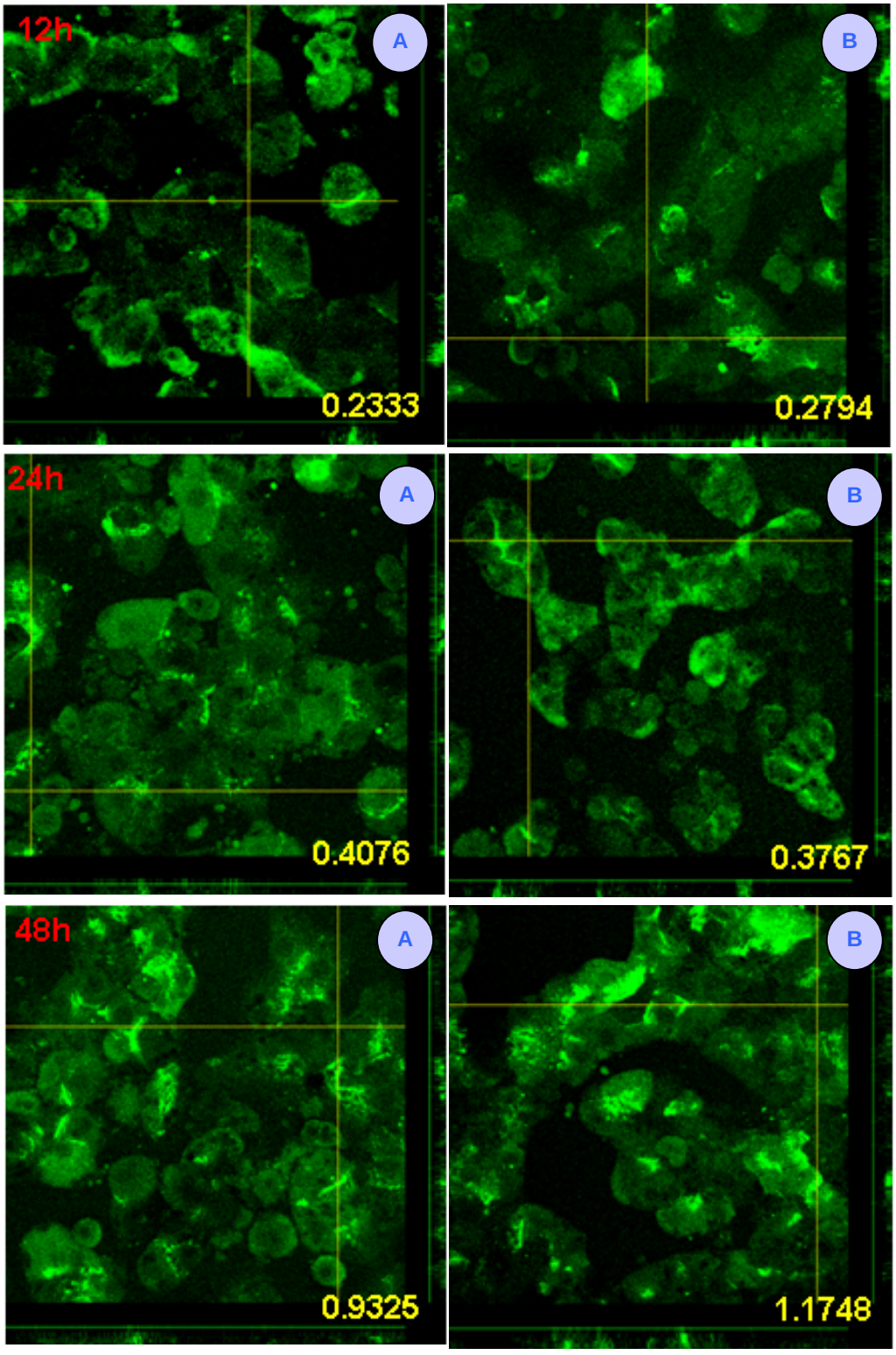
Fig 4.10 SEM pictures of hepatocytes cultured in the synthetic sandwich configuration with Gal at the bottom and RGD at the top (b) and in the conventional collagen sandwich (a).

4.4.2 Polarity genesis

As the building of cell polarity and functional activity of bile canaliculi can be represented by biliary excretion function of hepatocytes, we examined the kinetic changes of hepatocytes' excretory function in both sandwich configurations using a non-fluorescent substrate, fluorescein diacetate (FDA), which enters the cell by passive diffusion where it is hydrolyzed by intracellular esterases into fluorescein before it is excreted by bile canaliculi transporters. There was nearly no fluorescein concentrated in sacs between hepatocytes in conventional collagen sandwich after 12h of sandwich culture; and the fluorescein secreted by bile canaliculi begin to appear after 24h of culture and fully developed between 48h and 72h. However, in synthetic sandwich configuration, fluorescein secreted by bile canaliculi can be observed after 12h of sandwich culture; and the bile secretion increase with the time and higher level of fluorescein in sacs was developed between 48h and 72h of culture (Figure 4.11). This observation was further confirmed by quantifying the fluorescein localized in the inter-cellular sacs between hepatocytes and the absolute value was shown in bottom of each respectively pictures. The results suggest that hepatocytes cultured in synthetic sandwich configuration exhibited a same extent of functional polarity as conventional collagen sandwich.

4.4.3 Functional maintenance

We examined and compared the liver-specific functions of hepatocytes cultured in both sandwich configurations. Albumin secretion, urea production and 7-ethoxyresorufin-O-deethylation cytochrome P450 activity of hepatocytes cultured in synthetic sandwich configuration were significantly higher than those produce in the conventional collagen sandwich over 14 days of culture (Figure 4.12).


12h
A
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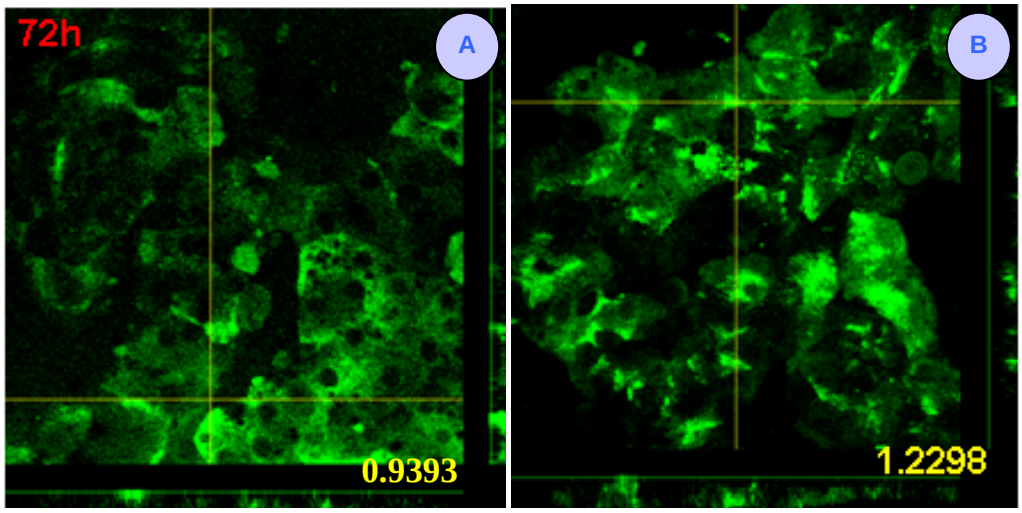
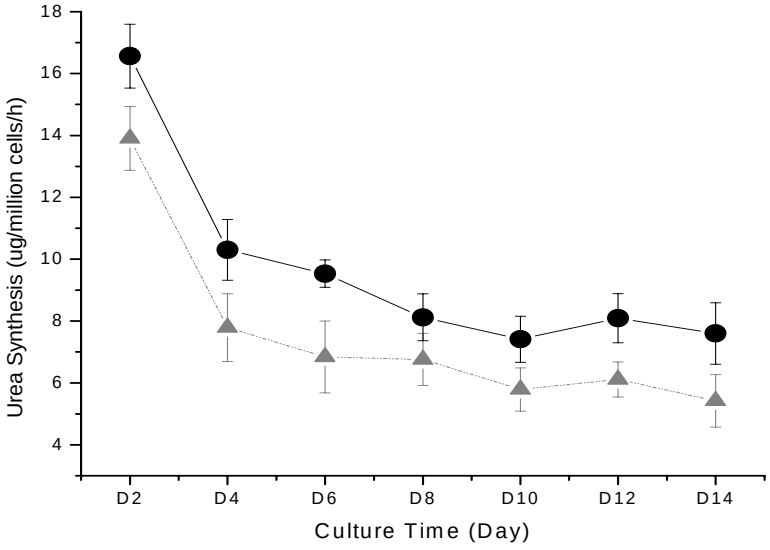
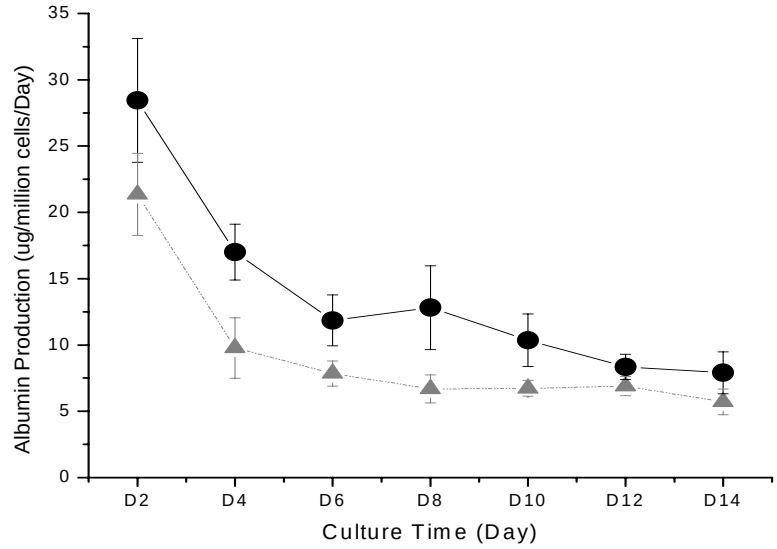


Fig 4.11 Excretory function of hepatocytes in the synthetic sandwich configuration (B) compared with the excretory function in conventional collagen sandwich (A) after different culture period after overlay.

The greatest enhancement was within the first 4 to 6 days, which may be translated into a higher level of functions upon stabilization. The improvement in the functional maintenance may be due to the better cell-cell interaction and improved mass transfer efficacy across the two layers of ECMs support in synthetic sandwich configuration.

Several system advantages have been demonstrated in the synthetic sandwich configuration over the conventional collagen, which may be related to the higher functional maintenance in synthetic sandwich configuration. When the cell-cell interaction was examined, a more tightly organized cell-cell interaction with featured cellular protrusions extending from one cell to another cell was observed in synthetic sandwich configuration over the conventional collagen sandwich. Cell-cell interaction has been shown to be essential for polarity formation and functional maintenance of hepatocytes, which may, from another point of view, demonstrate the ability of synthetic sandwich configuration in polarity formation and functional maintenance.


Urea Synthesis (ug/million cells/h)
18
16
14
12
10
8
6
4
D2
D4
D6
D8
D10
D12
D14
Culture Time (Day)


Albumin Production (ug/million cells/Day)
35
30
25
20
15
10
5
0
D2
D4
D6
D8
D10
D12
D14
Culture Time (Day)

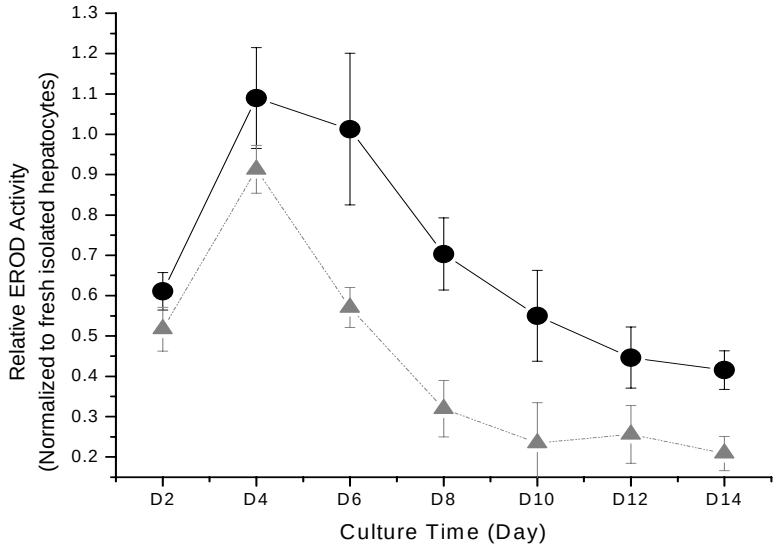


Fig 4.12 Functional maintenance of hepatocytes in the synthetic sandwich configuration in comaperation of hepatocytes' functions in conventional collagen sandwich in different culture days.

This better cell-cell interaction may partly result from the initial tighter cell-cell interaction of 3D monolayer before overlay. One of the inherent limitation of the conventional sandwich culture is the mass transfer barrier caused by the introduction of natural ECMs such as collagen type I, which slows down the exchange of nutrients, products, and chemical signals with the bulk of the medium and have been suggested to result in the decrease of hepatocytes functions. While polymeric materials with proper properties can help to improve. Higher mass transfer ability across the PET membrane was observed over collagen; another advantage of this synthetic system is based on the uniform functional modification of PET substrates. In addition, we observed that a more homogeneous cell morphology and structure were maintained in synthetic sandwich over collagen sandwich, which can be greatly affected by the coating uniformity and the unspecific cell-biomaterial interaction. With all those advantages including enhanced mass transfer efficacy, better cell-cell interaction and

higher initial functions before overlay, hepatocytes cultured in synthetic sandwich configuration exhibited improved albumin secretion, urea synthesis and P450 activities over conventional collagen sandwich for at least 14 days of culture. By regulating this 3D monolayer with proper chemical and physical overlay support (RGD- PET membrane), we formed the novel sandwich configuration with comparable performance to current collagen sandwich with similar polarity formation but better functional maintenance, which not only represents a novel sandwich culture configuration with improved hepatocytes performance for relevant applications in liver engineering, but also demonstrates the potentials of bio-synthetic matrix in tissue engineering applications.

Chapter 5 Conclusion and Future Work

The author characterized inherent mass transfer barriers in conventional sandwich configuration (indicated by albumin-rich intra-sandwich environment) and explored solutions to address this problem using two different bioengineering approaches.

As a positive influence of the flow environment in hepatocytes culture had been widely accepted for improving mass transfer, the effect of top flow conditions in sandwich configuration on mass transfer efficacy was tested in a flat-bed bioreactor. It was observed that mass transfer efficacy is hard to control in this way by varying the perfusion flow rates in perfusion culture without adversely affecting the hepatocytes functions. Thus, we designed a novel bioreactor with a separate drainage channel directly connected to the intra-sandwich environment to effectively regulate the intra-sandwich environment and allow exchange of nutrients and metabolites without changing the perfusion flow rates as is shown in Figure 2.1. An optimal level of the mass transfer efficacy can be maintained, which improved hepatocytes functions over the no-drainage controls for an extended period of time of up to 2 weeks.

The improved sandwich configuration developed under this strategy is valuable in the development of next generation of bio-artificial liver assistant device with the incorporation of novel excretion and drainage functions of bile production and toxins. The wide adoption of this approach for other applications is limited by the complexity of this system, which requires not only a successful bioreactor design and optimization of various flow environments based on individual cases with the uses of various regulation

pumps, but also big incubator as well as comprehensive flow circuits.

To the hepatic drug testing and high-throughput drug screening, obviously, we cannot use this complex system. This is the reason for us to focus on the simple strategies and try to improve the mass transfer efficacy by replacing the natural ECMs with the synthetic polymers of controllable physical and chemical properties. This simplifies the system complex and allows for scale up. After trying with functional polymers, we have created an ideal synthetic sandwich configuration by overlaying a novel 3D monolayer developed on galactosylated PET film with RGD conjugated polyethylene terephthalate membranes. This natural ECM-free sandwich configuration has been proved to possess higher mass transfer efficacy, improved functional maintenance and similar liver-specific functional structure compared with conventional sandwich configurations.

The interesting preliminary results validated the promising application of this system into hepatic drug testing without the incorporation of complicated natural ECMs and perfusion culture with improved system performance. Although the current configuration can not regulate the mass transfer efficacy to different degrees, the potential of this synthetic sandwich cannot be underestimated. With the rapid development of biomaterials, we definitely can produce the ideal functional layer for sandwich configuration uses with various physical and chemical properties such as pore size, matrix stiffness and the intensity and kinds of various ligands. This in turn, will tremendously enhances the performance of this synthetic sandwich configuration with controllable mass transfer efficacy.

References

[1] Langer, R, Vacanti, J.P. Tissue engineering. Science. 1993. 260 (14): p. 920–926

[2] Ghobrial, R.M. et al. Predictors of survival after *in vivo* split liver transplantation: Analysis of 110 consecutive patients. Ann. Surg. 2000. 232: p. 312-323.

[3] Starzl, T.E. Liver allo-transplantation and xenotransplantation. Transplant. Proc. 1993. 25: p. 15-24.

[4]: Vunjak-Novakovic, G. et al. Effects of mixing on the composition and morphology of tissue engineered cartilage. Am. Inst. Chem. Eng. J. 1996.42: p. 850–860.

[5]: Niclason, L.E. et al. Functional arteries grown *in vitro*. Science. 1999. 284: p. 489–493.

[6]: Guillouzo, A. Liver cell models in *in vitro* toxicology. Environ Health Perspect. 1998. 106 (Suppl 2): p. 511-532.

[7]: Ulrich, A. S., Vacanti, J.P. Tissue engineering: current state and prospects. Annu. Rev. Med. 2001. 52: p.443–451

[8]: Thomson, J.A., Itskovitz-Eldor, J., and Shapiro, S.S. Embryonic stem cell lines derived from human blastocysts. Science. 1998. 252: p.1145–1147.

[9] Hamazaki, T. et al. Hepatic maturation in differentiating embryonic stem cells *in vitro*. FEBS Lett. 2001. 497: p. 15-19.

[10]: Ai, Z. et al. Wnt-1 regulation of connexin43 in cardiac myocytes. J. Clin. Invest. 2000.105: p.161–171.

[11]: Courtman, D.W. et al. Development of a pericardial acellular matrix biomaterial: biochemical and mechanical effects of cell extraction. J. Biomed. Mater. Res. 1994.18: p. 655–666.

[12]: Vacanti, J.P., Morse, M.A., and Saltzman, W.M. et al. Selective cell transplantation using bioabsorbable artificial polymers as matrices. J. Pediatr. Surg. 1988. 23: P. 3–9.

[13]: Matsumoto, T. et al. The fate of the inverted segment of small bowel used for the replacement of major veins. Surgery. 1965. 60: p. 739–743.

[14]: Griffth. L.G. Ploymeric materials. Acta Mater. 2000. 48: p. 263-277.

[15]: Butler, D.L. et al. Functional tissue engineering: the role of biomechanics. J. Biomech. Eng. 2000. 122: p. 570–575.

[16]: Mazariegos, G.V. et al. First clinical use of a novel bioartificial liver support system (BLSS). Am. J. Transplant. 2002. 2: p.260–266.

[17]: Xiao, Y.L. et al. Static and dynamic fibroblast seeding and cultivation in porous PEO/PBT scaffolds. J. Mater. Sci. Mater.Med. 1999. 10: p. 773–777.

[18]: Wendt, D. et al. Oscillating perfusion of cell suspensions through three-dimensional scaffolds enhances cell seeding efficiency and uniformity. Biotechnol. Bioeng. 2003. 84: p.205–214.

[19]: Strain, A.J., Neuberger, J. M. A bioartifical liver − state of the art. Science. 2002. 295: p.1005-1009.

[20]: Strain, A. J., Diehl, A. Eds., Liver Growth and Repair (Chapman & Hall, London) 1998. p. 3-27 and 558-576.

[21]: Matthews, W.J., Conti, J.M., and Sireci, S.G. The effects of intercessory prayer, positive visualization, and expectancy on the well-being of kidney dialysis patients. Altern Ther Health Med. 2001. 7(5): p.42-52.

[22]: Rozga, J., et al. A Bioartificial liver to treat severe acute liver failure. Ann. Surg. 1994. 219: p. 538-546.

[23]: Nyberg, S.L. et al. Extracorporeal application of a gelentrapment, bioartificial liver: Demonstration of drug metabolism and other biochemical functions. Cell Transplant. 1993. 2: p. 441-449.

[24]: Gerlach, J.C. et al. Bioreactor for a larger scale hepatocyte *in vitro* perfusion. Transplantation. 1994. 58: p. 984-988.

[25]: Wolfe, S.P. et al. A novel multi-coaxial hollow fiber bioreactor for adherent cell types. Part 1: hydrodynamic studies Biotechnol. Bioeng. 2002. 77: p .83-90.

[26]: Ratcliffe A. Niklason, L.E. Bioreactors and Bioprocessing for Tissue Engineering. Annals of the New York Academy of Sciences. 2002. 961: p. 210-215.

[27]: Allen, J.W., and Bhatia, S.N. Engineering Liver Therapies for the Future. Tissue Engineering. 2002. 8(5): p.725-737.

[28]: Gion, T. et al. Evaluation of a hybrid artificial liver using a polyurethane foam packed-bed culture system in dogs. J. Surg. Res. 1999. 82: p.131-136.

[29] Doré, E. Legallais, C. A new concept of bioartificial liver based on a fluidized bed bioreactor. Ther. Apher. 1999. 3: p.264-278.

[30]: Kleinstreuer, C., Agarwal, S.S. Analysis and simulation of hollow-fiber

bioreactor dynamics. Biotechnol Bioeng. 1986. 28: p.1233-1240.

[31]: Griffith, L.G and Swartz, M.A. Capturing complex 3D tissue physiology *in vitro*. Nature Review. 2006. 7: p.211-224.

[32]: Khanna, H.J. et al. Polysaccharide scaffolds for hepatocyte transplantation: Design, seeding, and functional evaluation. Tissue Eng. 2000. 6: p.670-679.

[33]: Demetriou, A.A. et al. Transplantation of microcarrier-attached hepatocytes into 90% partially hepatectomized rats. Hepatology. 1988. 8: p.1006-1009.

.[34]: Karamuk, E. et al. Partially degradable film/fabric composites: Textile scaffolds for liver cell culture. Artif. Organs. 1999. 23: p.881-884.

[35]: Glicklis, R. et al. Hepatocyte behavior within three-dimensional porous alginate scaffolds. Biotechnol. Bioeng. 2000. 67: p.344-353.

[36]: Demetriou, A.A. et al. Survival, organization, and function of microcarrier-attached hepatocytes transplanted in rats. Proc. Natl. Acad. Sci.. 1986. 83: p.7475-7479.

[37]: Dixit, V. et al. Improved function of microencapsulated hepatocytes in a hybrid bioartificial liver support system. Artif. Organs. 1992. 16: p.336-341.

[38]: Saito, S., Sakagami, K., and Orita, K. A new hybrid artificial liver using a combination of hepatocytes and biomatrix. ASAIO Trans. 1987. 33: p.459-462.

[39]: Chang, T.M.S. Artificial liver support based on artificial cells with emphasis on encapsulated hepatocytes. Artif. Organs. 1992. 16: p.71-74.

[40]: Miura, Y. et al. Synthesis and secretion of protein by hepatocytes entrapped

within calcium alginate. Artif. Organs. 1986. 10: p.460-465.

[41]: Tompkins, R.G. et al. Enzymatic function of alginate immobilized rat hepatocytes. Biotechnol. Bioeng. 1988. 31: p.11-18.

[42]: Thompson, J.A. et al. Heparin- binding growth factor 1 induces the formation of organoid neovascular structures *in vivo* [published erratum appears in Proc. Natl. Acad. Sci. 1989. 86: p.7928-7932.

[43]: Ueno, K. et al. Formation of multicellular spheroids composed of rat hepatocytes. Res. Commun. Chem. Pathol. Pharmacol. 1992. 77: p.107-120.

[44]: Chang, T.M.S. Bioencapsulated hepatocytes for experimental liver support. J. Hepatol. 2001. 34: p.148-149.

[45]: Cai, A. et al. Development and evaluation of a system of microencapsulation of primary rat hepatocytes. Hepatology. 1989. 10: p.855-860.

[46]: Demetriou, A.A. et al. New method of hepatocyte transplantation and extracorporeal liver support. Ann. Surg. 1986. 204: p.259-271.

[47]: Kaihara, S., and Vacanti, J.P. Tissue engineering: Toward new solutions for transplantation and reconstructive surgery. Arch. Surg. 1999. 134: p.1184-1188.

[48]: Mooney, D.J. et al. Transplantation of hepatocytes using porous, biodegradable sponges. Transplant. Proc. 1994. 26: p.3425-3426.

[49]: Powers, M.J., Griffith, L.G. Adhesion-guided *in vitro* morphogenesis in pure and mixed cell cultures. Microsc. Res. Tech. 1998. 43: p.379-384.

[50]: Matsumoto, K. et al. Liver organogenesis promoted by endothelial cells prior to

vascular function. Science. 2001. 294: p.559-563.

[51]: Swales, N.J. Caldwell, J. Phase 1 and 2 Metabolism in Freshly Isolated Hepatocytes and Subcellular Fractions from Rat, Mouse, Chicken and Ox Livers. Pesticide Science. 1997. 49: p.291-299.

[52]: Paine, A.J., Hockin, L.J. The maintenance of cytochrome P-450 in liver cell culture: recent studies on P-450-mediated mechanisms of toxicity. Toxicology. 1982 25: p. 41-45.

[53]: Guillouzo, A. Liver cell models in *in vitro* toxicology. Environ Health Perspect. 1998. 106 Suppl (2): p. 511-532.

[54]: Ding, X. Kaminsky, L.S. Human extrahepatic cytochromes P450: Function in xenobiotic metabolism and tissue-Selective chemical toxicity in the respiratory and gastrointestinal tracts. Annual Review of Pharmacology and Toxicology, 2003. 43: p.149 – 173.

[55]: Thummel, K.E. Wilkinson, G. R. *In vitro* and *in vivo* drug interactions involving human CYP3A. Annual Review of Pharmacology and Toxicology. 1998. 38: P. 389-430

[56]: LeBlanc, G.A. Hepatic vectorial transport of xenobiotics. Chemico-Biological Interactions. 1994. 90: p. 101-120.

[57]: Turncliff, R.Z. et al. Hepatobiliary disposition of a drug/metabolite pair: Comprehensive pharmacokinetic modeling in sandwich-cultured rat hepatocytes.. J Pharmacol Exp Ther. 2006. 318(2): p. 881-889.

[58]: Chandra, P., Brouwer, K.L. The complexities of hepatic drug transport: current

knowledge and emerging concepts. Pharm Res. 2004. 21(5): p.719-735.

[59]: Moseley, R. H., Jarose, S. M., and Permoad, P. Organic cation transport by rat liver plasma membrane vesicles: studies with tetraethylammonium. Am J Physiol Gastrointest Liver Physiol. 1992. 263: p.775-785.

[60]: Borght, S.V. et al. Breast Cancer Resistance Protein (BCRP/ABCG2) Is Expressed by Progenitor Cells/Reactive Ductules and Hepatocytes and Its Expression Pattern Is Influenced by Disease Etiology and Species Type: Possible Functional Consequences. Journal of Histochemistry and Cytochemistry. 2006. 54 (9): p.1051-1059.

[61]：Coleman, R., Roma, M. G. Biochemical Society Transactions. 2000. 28: p.136-140.

[62]: Roskelley, C. D., Desprez, P. Y. and Bissell, M. J. Extracellular matrix-dependent tissue-specific gene expression in mammary epithelial cells requires both physical and biochemical signal transduction. Proc. Natl Acad. Sci. 1994. 91: p.12378–12382.

[63]: Grinnell, F. et al. Differences in the regulation of fibroblast contraction of floating versus stressed collagen matrices. J. Biol. Chem. 1999. 274: p.918-923.

[64]: Choa, C.S.. Galactose-carrying polymers as extracellular matrices for liver tissue engineering. 2006. 27 (4): p.576-585.

[65]: Baatout S. Endothelial differentiation using Matrigel. Anticancer Res. 1997. 17(1A): p.451-455.

[66]: Bader, A. et al. Development of a small-scale bioreactor for drug metabolism studies maintaining hepato-specific functions. Xenobiotica. 1998. 28(9): p.815-825.

[67]: LeCluyse, E.L., Audus, K.L., and Hochman, J.H. Formation of extensive canalicular networks by rat hepatocytes cultured in collagen-sandwich configuration. Am. J. Physiol. 1994. 266(1):p. 1764-1774.

[68]: Brown, L.A. et al. Maintenance of liver functions in rat hepatocytes cultured as spheroids in a rotating wall vessel. *In Vitro* Intra-sandwich & Developmental Biology – Animal. 2003. 39 (1): p.13-20.

[69]: Bhandari. R.N. et al. Liver tissue engineering: a role for co-culture systems in modifying hepatocyte function and viability. Tissue Eng. 2001.7(3): p. 345-357.

[70]: Bader. A. et al. 3-D coculture of hepatic sinusoidal cells with primary hepatocytes-design of an organotypical model. Exp Cell Res. 1996. 226(1): p.223-233.

[71]: Bailly-Maitre, B. et al. Dexamethasone inhibits spontaneous apoptosis in primary cultures of human and rat hepatocytes via Bcl-2 and Bcl-xL induction. Cell Death Differ. 2001, 8(3): p.279-288.

[72]: Mitaka T. et al. Subculture of proliferating adult rat hepatocytes in medium supplemented with nicotinamide and EGF. *In Vitro* Cell Dev Biol Anim. 1996. 32(8): p.469-477.

[73]: Ivan, M. David, W. and Michael, H. The role of bioreactors in tissue engineering.

Trends Biotechnol. 2004. 22(2): p. 80-86.

[74]: Ledezma, G.A. et al. Numerical model of fluid flow and oxygen transport in a radial-flow microchannel containing hepatocytes. J. Biomech. Eng. 1999. 121: p. 58-64.

[75]: Gerlach, J.C. et al. Improved hepatocyte in-vitro maintenance in a culture model with woven multicompartment capillary systems—electron microscopy studies. Hepatology. 1995. 22: p.546-552.

[76]: Wu, F.J. et al. Entrapment of hepatocyte spheroids in a hollow fiber bioreactor as a potential bioartificial liver. Tissue Eng. 1995. 1: p.29-35.

[77]: Sakai, Y. et al. A new bioartificial liver using porcine hepatocyte spheroids in high-cell-density suspension perfusion culture: *in vitro* performance in synthesized culture medium and in 100% human plasma. Cell Transplant. 1999. 8: p.531-541.

[78]: Koide, N. et al. Continued high albumin production by multicellular spheroids of adult rat hepatocytes formed in the presence of liver-derived proteoglycans. Biochem Biophys Res Commun. 1989. 161: p.385-391.

[79]: Powers, M.J. Griffith, L.G. Cell-substratum adhesion strength as a determinant of hepatocyte aggregate morphology. Biotechnology and Bioengineering. 1997. 53: 415-426.

[80]: Ying, L. et al. Immobilization of galactose ligands on acrylic acid graft-copolymerized poly(ethylene terephthalate) film and its application to hepatocyte culture, Biomacromolecules. 2003, 4: p.157–165.

[81]: Walker, T.M., Rhodes, P.C., and Westmoreland, C. The differential cytotoxicity of methotrexate in rat hepatocyte monolayer and spheroid cultures. Toxicol *In Vitro*. 2000;14(5): p.475 – 485.

[82]: Yasuyuki, S. et al. Quantitative comparison of rat hepatocyte functions in two improved culture systems with or without rat liver epithelial cell line. Cytotechnology. 1996. 21(3): p.243 – 252.

[83]: Bhatia, S.N. et al. Controlling cell interactions by micropatterning in co-cultures: Hepatocytes and 3T3 fibroblasts. Journal of Biomedical Materials Research, 1997. 34: p.189–199.

[84]: Dunn, J.C.Y. et al. Hepatocyte function and extracellular matrix geometry: long-term culture in a sandwich configuration. FASEB J. 1989. 3: p.174-177.

[85]: Dunn, J. C. Y., Tompkins, R. C., and Yamsush, M. L. Long-term *in vitro* function of adult hepatocytes in a collagen sandwich configuration. Biotechnol. Prog. 1991. 7: p.237-245.

[86]: Moghe,P. V. et al. Role of extracellular matrix composition and configuration in maintenance of hepatocyte polarity and function. BiomaseriaL, 1996. 17: p.373-385.

[87]：Ezzell, R. M. et al. Effect of collagen gel configuration on the cytoskeleton in cultured rat hepatocytes. Exp. Cell Res. 1993. 208: p.442-452.

[88]: Liu, X. et al. Biliary excretion in primary rat hepatocytes cultured in a collagen-sandwich configuration. Am. J. Physiol. 1999. 277: p.12-21.

[89]: Ghibellini, G. et al. Methods to evaluate biliary excretion of drugs in humans: an

updated review. Mol Pharm. 2006. 3: p.198-211.

[90]: Suzuki, T., Nishio, K., and Tanabe S. The MRP Family and Anticancer Drug Metabolism. Current Drug Metabolism. 2001. 2: 367-377.

[91]: Yeaman, C., Grindstaff, K.K., and Nelson, W.J. New Perspectives on Mechanisms Involved in Generating Epithelial Cell Polarity. Physiological Rev. 1999. 79(1): p.79-.

[92]: Musat, A. I. et al. Reestablishment of cell polarity of rat hepatocytes in primary culture. Hepatology. 1993.18: p.198-205.

[93]: Dunn, J., Tompkins, R. C., and Yarmush, M. L. Hepatocytes in collagen sandwich configuration: evidence for transcriptional and translational control. J. Cell Biol. 1992. 116: p.1043-1053.

[94]: Sidhu, S., Farm, F.M., and Omiecinski, C. Influence of extracellular matrix overlay on phenobarbital.mediated induction of CYP2B1,282, and3A1 genes in primary adult rat hepatocyte culture. Arch. Biochem. Biophys. 1993. 301: p.103-113.

[95]: Han, X. et al. Binding of perfluorooctanoic acid to rat and human plasma proteins. Chem Res Toxicol. 2003.16(6): p.775-781.

[96]: Kragh-Hansen, U., Chuang, V.T. and Otagiri, M. Practical aspects of the ligand-binding and enzymatic properties of human serum albumin. Biol Pharm Bull. 2002. 25(6): p.695-704.

[97]: Keller, K.H., Canales, E.R., and Yum, S.I. Tracer and mutual diffusion coefficients of proteins. J. Phys. Chem. 1971. 75: p.379-387.

[98]: Ekani-Nkodo, A., Fygenson, D.K. Size exclusion and diffusion of fluoresceinated probes within collagen fibrils. Phys Rev E. 2003. 67: p.1-7.

[99]: Shaw, M., Schy, A. Diffusion coefficient measurement by the "stop-flow" method in a 5% collagen gel. Biophys J. 1981. 34(3): p.375-381.

[100]: Sutherland, R.M. et al. Oxygenation and differentiation in multicellular spheroids of human colon carcinoma. Cancer Res. 1986. 46: p.5320–5329.

[101]: Ishaug, S.L. et al. Bone formation by three-dimensional stromal osteoblast culture in biodegradable polymer scaffolds. J. Biomed. Mater. Res. 1997. 36: p.17–28.

[102]: Gooch, K.J. et al. Effects of mixing intensity on tissueengineered cartilage. Biotechnol. Bioeng. 2001. 72: p.402–407

[103]: Vunjak-Novalovic, G. et al.Bioreactor cultivation conditions modulate the composition and mechanical properties of tissueengineered cartilage. J. Orthop. Res. 1999. 17: p.130–138

[104]: Pei, M. et al. Bioreactors mediate the effectiveness of tissue engineering scaffolds. FASEB J. 2002. 16: p.1691–1694.

[105]: Unsworth, B.R. and Lelkes, P.I. Growing tissues in microgravity. Nat. Med. 1998. 4: p.901–907.

[106]: Bancroft, G.N. et al. Fluid flow increases mineralized matrix deposition in 3D perfusion culture of marrow stromal osteoblasts in a dose-dependent manner. Proc. Natl. Acad. Sci. 2002. 99: p.12600–12605.

[107]: Goldstein, A.S. et al. Effect of convection on osteoblastic cell growth and

function in biodegradable polymer foam scaffolds. Biomaterials. 2001. 22: p.1279–1288.

[108]: Navarro, F.A. et al. Perfusion of medium improves growth of human oral neomucosal tissue constructs. Wound Repair Regen. 2001. 9: p.507–512.

[109]: Carrier, R.L. et al. Perfusion improves tissue architecture of engineered cardiac muscle. Tissue Eng. 2002. 8: p.175–188.

[110]: Pazzano, D. et al. Comparison of chondrogenesis in static and perfused bioreactor culture. Biotechnol. Prog. 2000. 16: p.893–896.

[111]: Fiegel, H.C. et al. Influence of flow conditions and matrix coatings on growth and differentiation of three-dimensionally cultured rat hepatocytes. Tissue Eng. 2004. 10: p.165-174.

[112]: Park, J., Berthiaume et al. Microfabricated Grooved Substrates as Platforms for Bioartificial Liver Reactors. Biotechnol Bioeng. 2005. 90(5): p.632-644.

[113]: Tilles, A.W. et al. Effects of oxygenation and flow on the viability and function of rat hepatocytes cocultured in a microchannel flat-plate bioreactor. Biotechnol Bioeng. 2001. 73(5): p.379-389.

[114]: Lalan, S. et al. Tissue Engineering and Its Potential Impact on Surgery. World Journal of Surgery. 2004. 25: p. 1458-1466.

[115]: LeCluyse, E.L. et al. Cultured rat hepatocytes. Pharm Biotechnol 1996. 8: p.121-159.

[116]: Lu H.F. et al. Galactosylated PVDF membrane promotes hepatocyte attachment and functional maintenance. Biomaterials. 2003. 24(27): p.4893 – 4903.

[117]: Tashiro, K. et al. The RGD containing site of the mouse laminin A chain is active for cell attachment, spreading, migration and neurite outgrowth. J Cell Physiol 1991. 146(3): p.451 – 459.

[118]: Carlisle, E.S. et al. Enhancing hepatocyte adhesion by pulsed plasma deposition and polyethylene glycol coupling. Tissue Eng. 2000. 6(1): p.45 – 52.

[119]: Weigel, P.H et al. Adhesion of hepatocytes to immobilized sugars, A threshold phenomenon. J Biol Chem. 1979. 254: p.10830–10838.

[120]: Weigel, P.H et al. Specific adhesion of rat hepatocytes to beta-galactosides linked to polyacrylamide gels, J Biol Chem. 1978. 253: p.330–333.

[121]: Weisz, O.A. Schnaar, R.L. Hepatocyte adhesion to carbohydrate-derivatized surfaces. I. Surface topography of the rat hepatic lectin, J Cell Biol. 1991.115: p. 485–493.

[122]: Kobayashi, K. Kobayashi, A. and Akaike,T. Culturing hepatocytes on lactose-carrying polystyrene layer via asialoglycoprotein receptor-mediated interactions. In: Y.C. Lee and R.T. Lee, Editors, Methods in Enzymology: Neoglycoconjugates part B Biomedical Applications Vol. 247, Academic Press, San Diego, California. 1994. p. 409–418.

[123]: Tobe, S. Y. et al. Receptor-mediated formation of multilayer aggregates of primary cultured adult rat hepatocytes on lactose-substituted polystyrene, Biochem Biophys Res Commun. 1992. **184**: p. 225–230.

[124]: Lopina,S.T. et al. Hepatocyte culture on carbohydrate-modified star

polyethylene oxide hydrogels, Biomaterials. 1996. **17**: p. 559–569.

[125]: Yin, C. et al. High density of immobilized galactose ligand enhances hepatocyte attachment and function, J Biomed Mater Res A. 2003. **67**: p. 1093–1104.

[126]: Kang, I.K. et al. Grafting of lactose-carrying styrene onto polystrene dishes using plasma glow discharge and their interaction with hepatocytes, J Mater Sci Mater Med. 2003. **14**: p. 611–616.

[127]: Wallner, E.I. et al. Relevance of extracellular matrix, its receptors, and cell adhesion molecules in mammalian nephrogenesis. Am J Physiol Renal Physiol. 1998. 275: p.467-477.

[128]: Du, Y. et al. 3D hepatocyte monolayer on hybrid RGD/galactose substratum. Biomaterials 2006. 27: p.5669-5680.

[129]：Lee. J. M, J.R., Tompkins, R.G., Yarmush. M. L. Proline-mediated enhancement of hepatocyte function in a collagen gel sandwich culture configuration. FASEB J. 1993. 7: p.586-591.

[130]: Ng, S. et al. Improved hepatocyte excretory function by immediate presentation of polarity cues. Tissue Eng. 2006. In Press.

[131]: Van Damme, M.P., Comper, W.D., and Preston, B.N. Experimental measurements of polymer unidirectional fluxes in polymer + solvent systems with non-zero chemical-potential gradients. J. Chem. Soc. Faraday Trans. 1982. 78: p.3357-3367.

[132]: Dixit, V., Chang, T.M.S. *In vitro* and clinical studies of the removal of

cortisol, thyroxine, insulin, and thyroid-stimulating hormone by coated charcoal haemoperfusion. *Life Support Syst*. 1984. 2(4): p.238-244.

[133]: Zamek-Gliszczynski, M.J. et al. Pharmacokinetics of 5 (and 6)-carboxy-2',7'-dichlorofluorescein and its diacetate promoiety in the liver. J. Pharmacol. Exp. Ther. 2003. 304(2): p. 801-809.

[134]: Seglen, P.O. Preparation of isolated rat liver cells. Methods Cell Biol. 1976. 13: p.29-83.

[135]: Gupta, B. et al. Plasma-induced graft polymerization of acrylic acid onto poly(ethylene terephthalate) films: characterization and human smooth muscle cell growth on grafted films. Biomaterials. 2002. 23(3): p.863 – 871.

[136]: Uchida YU E, Ikada Y. Sorption of low-molecular-weight anions into thin polycation layers grafted onto a film. Langmuir. 1993. 9: p.1121 – 1124.

[137]: Findeis, M.A. Stepwise synthesis of a GalNAc-containing cluster glycoside ligand of the asialoglycoprotein receptor. Int J Pept Protein Res 1994. 43(5): p.477 – 485.

[138]: Weigel, P.H. et al. Preparation of 6-aminohexyl D-galactopyranosides. Carbohydr Res. 1979. 70: p.83 – 91.

[139]: Dong, L.C., Hoffman, A.S., and Yan, Q. Dextran permeation through poly(N-isopropylacrylamide) hydrogels. J. Biomater. Sci. Polym. 1994. 5(5): p.473-484.

[140]: Liu, X. et al. Correlation of biliary excretion in sandwich-cultured rat hepatocytes and *in vivo* in rats. Drug Metab. Dispos. 1999. 27: p.637-644.