CHAPTER 8

Bioreactors for Liver Tissue Engineering

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Summary

Acute liver failure (ALF) patients have a high risk of mortality. The elective treatment for ALF still is orthotopic liver transplantation. Donor organ scarcity and the associated high costs make transplantation possible for only about one third of the patients on the transplantation waiting list. Engineering a biological liver substitute is an interesting alternative to the traditional ALF therapy. Cells seeded in three-dimensional scaffolds made of bioresorbable biomaterials might be implanted in vivo to replace part of, or the whole, liver and integrate with time. Two- or three-dimensional non-implantable constructs may be used ex vivo for the EC support of ALF patients until a tissue compatible organ is available or the patient's own liver heals. Non-implantable three-dimensional constructs may be also useful to investigate liver cell metabolism or as an alternative to animal tests for drug screening or toxicity assessment in vitro. Over the years, research has mainly focused on the development of new culture techniques or new immortalized hepatic cell lines. A major obstacle to the generation of functional substitutes is the limited understanding of the role of specific physical-chemical parameters on tissue development. Bioreactors provide controlled environmental conditions to improve the quality of tissue and to study liver cell metabolism. This review focuses on the bioreactors that have been proposed to develop enhanced biological liver substitutes or study liver cell metabolism.

Keywords: Bioreactors; Cell; Liver; Metabolism; Tissue engineering

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Introduction

Acute liver failure (ALF) caused by drug poisoning, surgical complications, viral infections or decompensated chronic liver disorders has a high risk of mortality. ALF may progress within a few weeks from the first symptoms (e.g., increased ammonia levels or the onset of jaundice) onto life threatening complications (e.g., generalized oedema, bleeding and hepatic encephalopathy) that culminate in multiorgan failure and death [1]. ALF has a poor prognosis under conservative management, with survival rates ranging from 79% (for *amanita* intoxication) to 10% (cryptogenic genesis) depending on its etiology [2]. The elective treatment for ALF is orthotopic liver transplantation (OLTx) with survival rates up to 60-80% [3]. Until 2002, about one in three patients died while on the transplant waiting list and the number of donor organs keeps decreasing since [4]. Donor organ shortage and the high social costs of transplantation and the associated year-long immunosuppressive therapy limit the number of liver transplants. However, liver tissue has the potential to regenerate and heal [5].
ALF is generally associated with the accumulation of plasmatic toxins (such as ammonia, mercaptans, free phenols, bile acids, benzodiazepines, etc.) [6]. Thus, over the years, many extracorporeal (EC) artificial liver support devices have been proposed for the treatment of ALF, or to bridge a patient to OLTx in good mental conditions, that have not brought about significant improvements over conventional patient management. In these devices, perm-selective membranes or adsorption cartridges are used to simulate the liver detoxification functions and remove from the blood some of the putative toxins damaging the liver. More advanced systems have been reported to remove also protein-bound toxins by transferring them to an albumin-rich solution across porous polysulphone membranes [7-9]. In one of them, albumin is stably adsorbed in the membrane pores [7]. In these devices, the stripping albumin solution is continuously regenerated over an adsorption column or goes to waste. Interesting results have been reported in the treatment of acute-on-chronic (AoC) hepatic patients, although the efficacy of these devices has not yet been proven in blind trials.
The need of liver-specific metabolic products to relieve some of the symptoms (e.g., cerebral oedema and bleeding) and promote liver tissue regeneration started the quest for alternative cell-based therapies aimed at providing ALF patients also with the liver biosynthetic and biotransformation functions.

Tissue engineering (TE) is a multidisciplinary science aimed at developing “biological substitutes to restore, maintain or augment tissue function” [10], and holds promise for the

development of innovative alternatives to ALF treatment. The ideal biological liver substitute should perform most or all of the liver-specific detoxification, synthetic and biotransformation functions. As most of these functions are still unknown, mature liver cells (e.g., primary or immortalized) or cells that may differentiate into hepatocytes (e.g., stem or progenitor oval cells) have been used in these substitutes to perform liver-specific functions. Typically, liver constructs are engineered *in vitro* by culturing liver (or liver-like) cells in/on synthetic scaffolds which provide the template for cell adhesion, re-arrangement, proliferation and development. Three-dimensional (3D) implantable constructs are made of porous biomaterials that degrade and resorb at controlled rates to permit their replacement with the extracellular matrix (ECM) produced by the cells, and cell colonization of the construct. Once implanted *in vivo*, the graft has to fully integrate into the body of the host. Two-dimensional (2D) or 3D non-implantable constructs may be used *ex vivo* for the EC support of ALF patients till a tissue compatible organ is available or the patient's own liver heals. These constructs are expected to provide the patients only with the relevant liver functions that stop progression of the damages and promote liver regeneration, and to function for a limited time from a few weeks to a few months. Non-implantable constructs may be also useful to investigate liver cell metabolism or as an alternative to animal tests for the *in vitro* drug screening or toxicity assessment. In this case, the cells and the device in which these constructs are used, as a whole, should express enzymatic activities and yield metabolite profiles similar to those in the natural liver for at least a few months to represent a feasible and convenient alternative to animal tests.

The development of constructs with metabolic functions equivalent to those of the liver poses technical challenges well beyond that of developing new culture techniques (e.g., 2D monolayer vs. 3D culture) for the complexity of liver cell physical-chemical requirements and the scale of the constructs. In fact, the liver is a highly structured organ with many distinct cell sub-populations. In the liver parenchyma, the hepatocytes perform most of the liver-specific metabolic functions. Hepatocytes are arranged in plates in repeating units termed "lobules" in which they spread outward from a central vein. At the lobule vertices, a bile duct and a branch of the hepatic artery and portal vein are located close to one another in an arrangement often termed "the portal triads". Blood flows from the two vessel branches towards the central vein through small vascular channels, termed "sinusoids", lined with a fenestrated layer of endothelial cells. Plasma filters through the endotelium into the space that separates it from the hepatocytes (i.e., the space of Disse), and exchanges nutrients and metabolites with the hepatocytes through their apical surface. Bile is secreted into the *canaliculi* formed between the

basal surfaces of adjacent hepatocytes, and flows through the bile ducts into the common bile duct that dumps it into the duodenum. Kuppfer cells, ECM-producing stellate cells, biliary epithelial cells, hepatocyte precursor cells and fibroblasts are also present and perform important metabolic functions [11]. Liver cells are spatially organized to optimize communication and transport. Cells communicate directly through cellular and gap junctions, and via chemical signals dissolved and blood-borne or present in the macromolecules forming the ECM that surrounds them. The signals that cells exchange promote differentiation, proliferation and functions [12-14]. Furthermore, metabolic (e.g., carbohydrate metabolism) and detoxification (e.g., CYP450 enzymes) activities of the hepatocytes change spatially along the length of the sinusoid, apparently regulated by gradients of oxygen, hormones and ECM composition, a phenomenon termed “liver zonation” [15,16]. Information on the structure-function relationship for normal and pathological liver tissue is still lacking. Fostering the same cellular relationships existing in the normal liver also in the TE liver construct is considered fundamental for cells to function as in the natural liver. This has suggested to pattern the biological substitutes after the liver micro-architecture, although reproducing the whole liver architecture in constructs for EC liver support might not be necessary for cells to perform only the subset of hepatic functions relevant to stop progression of ALF. Hepatectomy studies suggest that TE liver constructs should perform metabolic functions quantitatively equivalent to at least 30% of the natural liver mass to be effective in the treatment of ALF [5], and set the scale of the construct at one hosting an average of at least 500 g cells. An additional complication is that the engineering of liver tissue *in vitro* occurs through processes whose length and time scale spans over orders of magnitude. In fact, cells adhere on the biomaterial surface by recognizing morphological and biochemical patterns in the nanometer range but have to organize in hierarchical structures a few centimeters large. Proteins from serum adsorb on, and mediate cell interactions with, the biomaterial surface within milliseconds but it may take weeks before liver cells differentiate and re-arrange in functional liver-like cellular structures [7]. That said above clearly outlines the formidable technical challenges posed by liver tissue engineering. Over the years, research has mainly focused on the procurement of large amounts of suitable liver cells and the development of *in vitro* culture techniques [17-19]. Increasing research effort is being devoted to the development of biomaterials for liver cell scaffolding at whose surface topological, morphological and biochemical signals are present to attract cells and make them adhere and rearrange their cytoskeleton, and to the characterization of the dissolved biochemical and physical signals that control cell differentiation, proliferation and apoptosis [20].

This chapter focuses on the bioreactors proposed for liver tissue engineering. A bioreactor may possibly be defined as a volume (or vessel) in which one or more biochemical or biological processes take place. Indeed, bioreactors are extensively used at any step of the assembly of a TE liver construct. In fact, primary cells enzymatically isolated from autogenous or allogeneic liver tissue (or the whole organ) are often cultured in Petri dishes or T-flasks prior to their seeding in the scaffold to let them recover from the isolation and purification stress. Immortalized and primary progenitor or stem liver cells are generally expanded in Petri dishes or T-flasks, often under conditions facilitating differentiation to mature hepatocytes of the first two cell types, or inhibiting differentiation to maximize proliferation of the third cell type. Liver cells (often of different types) are harvested and seeded in/on synthetic scaffolds in bioreactors under conditions that should facilitate cell attachment to the scaffold surface and penetration into its pores. The cell-seeded scaffolds are then cultured in bioreactors under tightly controlled and closely monitored environmental conditions to provide cells with biochemical and physical cues that should promote cell reorganization into liver-like aggregates and differentiation to make the construct functionally equivalent to liver tissue. Non-implantable constructs used for EC liver support are eventually cultured in bioreactors under conditions that maximize their therapeutic efficacy, prevent their rejection (if allogeneic cells are used) and intoxication caused by the ALF plasma, and preserve the liver cell phenotype for the treatment time. Non-implantable constructs used for metabolic investigation are eventually used in bioreactors under controlled, measurable and reproducible metabolite or drug concentrations to study liver cell metabolic behavior and differentiation. Below, the problems in bioreactor design for liver tissue engineering are briefly discussed and the proposed bioreactors presented.

Bioreactor design issues

The first step in the development of constructs for liver replacement is to seed a large mass of liver cells uniformly on, or throughout, a scaffold. Then, the adherent cells have to be provided with adequate amounts of oxygen and nutrients to survive and proliferate, and adequate biochemical/physical signals to re-organize and differentiate to yield cellular structures and metabolic zonation resembling that of the liver. Attaining adequate cell seeding, nutrient and oxygen supply to cells, and the control of biochemical signals gradients and concentrations in large scaffolds is not easy and depends on the bioreactor configuration and operation, on how

mass is transported outside and inside the scaffold, and on cell metabolism. Bioreactors used for extracorporeal liver support have also to be connected to the patient so as to guarantee the unhindered transport of soluble species from the patient's circulation to the cells in the bioreactor and vice versa. Below, this last issue shall only be briefly discussed.

Cell seeding

Cell seeding is thought to play a critical role in the development of *in vitro* engineered tissue. Seeding cells at high initial density may favor tissue formation [21]. High seeding efficiency would also limit the amount of organ tissue from which primary cells are isolated and cell expansion. Uniform initial cell distribution on 2D or throughout 3D scaffolds has been related to the uniformity of engineered cartilage and bone tissue [22-24]. Uneven cell distribution in the scaffold might lead to spatial variations in nutrients, oxygen and metabolite concentrations that would condition the survival and metabolism of cells at different positions in the scaffold. Seeding cells efficiently and uniformly on/in a scaffold is challenging, in particular throughout 3D porous scaffolds. In fact, closed or tight pores inside the scaffold may limit cell access and the seeding efficiency and distribution. Cell distribution in accessible pores depends on the balance between the rate at which cells are physically transported from the medium bulk to the outer scaffold surface (i.e., external transport) and from there towards its innermost pores (internal transport), and the rate at which cells bind to the biomaterial pore surface or to other cells in the feed suspension and form clusters. Resistance to external transport and cell uniformity in the feed suspension may be varied by changing the mixing intensity in the bioreactor where seeding takes place. Resistance to internal transport depends on the cell-to-pore size ratio but also on the transport mechanism within the scaffold. In fact, drag associated with medium convection through the pores enhances cell transport as compared to gravity or capillary forces only, although to an extent that depends on the actual pore size distribution and tortuosity of the scaffold. Cell association in clusters increases the size of the transported aggregate and limits, or hinders, their mobility within 3D scaffolds. Fast cell adhesion to the scaffold surface closer to its external surface may reduce the effective pore size and hinder farther cell penetration into 3D porous scaffolds.

Nutrient and metabolite transport

Growing liver tissue *in vitro* for liver replacement or support is more difficult than other tissues. In fact, cells have to be cultured at the high density typical of the natural liver, much

higher than in many other tissues. Liver cells have also important nutrient requirements and are sensitive to waste metabolites [25-29]. *In vivo*, the liver is efficiently provided with soluble nutrients by a high blood flow that reaches the innermost cells in the organ by means of a fine network of capillaries (i.e., the sinusoids). This keeps the diffusion distance between cells and the blood small (i.e., within a few hundreds micron). Providing an analogous system to supply basic substrates (e.g., oxygen, glucose and amino acids) to or clear waste metabolites (e.g., CO_2, ammonia, urea, lactate) from liver cells in large 3D constructs is a formidable challenge, and a pre-requisite to promote cell growth, differentiation and long-term survival [30-33]. In fact, nutrients are continuously consumed (and products formed) by the cells while they are transported from the source into the cell mass (or from the cells to the sink). This causes the concentration of soluble nutrients in the cell mass to be generally less than that near their source (for neo-synthesized species it would be higher than that near the sink), and the formation of concentration gradients across the cell construct. These gradients may induce different cell behaviors at different spatial positions in the construct, and even induce chemotaxis if the gradients are steep enough to be sensed by the cells [34]. Although not basic for cell survival, transport of large proteins may also have important effects on cell behavior and the therapeutic efficacy of constructs used in bioartificial livers (BALs) for EC liver support. In fact, their mobility is significantly lower than that of small nutrients. This causes a mass transport resistance that may substantially reduce the concentration of proteic effectors (e.g., EGF) with respect to their value in the feed medium and may hinder transport of neo-synthesized liver-specific proteins (e.g., clotting proteins and growth factors) back into the patient's blood. Three compartments may be identified in the bioreactors proposed for the *in vitro* culture of liver cell-seeded constructs: the medium compartment, where synthetic culture medium, plasma or blood from the patient's circulation flows; the membrane compartment, when membranes are used as immuno-selective barriers to protect the cells from rejection or to scaffold the cells; the cell compartment, where cells are seeded and cultured attached to a biocompatible scaffold or microcarrier, or in suspension. To reach the cells in the construct, soluble nutrients have to be transported across each compartment, in particular: external to the construct, from the medium bulk to the membrane (or the construct) outer surface; across the membrane wall; inside the construct, from the membrane inner (or the construct outer) surface into the cell construct across the cell mass. The spatial profile of soluble nutrients and wastes in the construct, and the rate at which they are transferred from one compartment to the other, depends on the mass transport resistance of each compartment. In the following, the factors affecting the resistance in each compartment are shortly presented and discussed as a means to

understanding the rationale of the different bioreactors proposed to optimize the *in vitro* culture of liver cells in large constructs. For the sake of the example, reference is made to the transport of oxygen, an important nutrient playing a key role in all aerobic metabolic processes, often limiting cell functions and tissue growth *in vitro*. For additional information, the Reader is referred to other available reviews on mass transport in bioreactors for tissue culture [30-34].

External transport – In conventional batch bioreactors (e.g., Petri dishes or T-flasks) for 2D cell or tissue culture, soluble species are transported by diffusion from the medium bulk to the cell surface across a stagnant layer of medium. When oxygen is supplied from the gas above the medium (as is often the case), the dissolved oxygen concentration at the gas-medium interface C_{eq} is limited by the scarce oxygen solubility in aqueous media. In fact, medium equilibrated with air would contain only ca. 0.2 $mmol_{oxygen}$/L. The actual dissolved oxygen concentration at the cell surface $C_{surface}$ is generally lower than C_{eq}. In particular, $C_{surface}$ increases with increasing C_{eq} (hence the gaseous O_2 partial pressure) and oxygen diffusivity in the medium D (ca. 2 x 10^{-5} cm^2/s), and decreases with increasing medium height above the cells h and cell oxygen consumption rate OCR. The balance at the liver cell surface of the oxygen diffusive flux (i.e., $D\ (C_{eq} - C_{surface})/h$) and the cellular rate of oxygen consumption yields oxygen concentrations at the cell surface decreasing from ca. 50 to 3% of the concentration at the gas-medium interface as the medium height above the cells increases from 0.5 to 2 mm (i.e., for $OCR=V_{max}\ C_{surface}/(K_m + C_{surface})$ and $V_{max}=k_{oxy}\ C_{cell}$ with k_{oxy}=0.38 nmol/10^6 cells/s, K_m=6.6 nmol/mL [28], for a confluent cell monolayer with ca. $C_{cell}=10^5$ cells/cm^2).

Culturing large cell-seeded 3D scaffolds in stagnant medium (e.g., in Falcon tubes) may result in even steeper spatial oxygen concentration gradients along the construct length. This causes the dissolved oxygen concentration to decrease significantly at zones of the outer construct surface farther away from the gaseous oxygen source.
External mass transport resistance can be reduced by superimposing convective transport to pure diffusion to increase the mixing. Under these conditions, the rate of solute transport to/from the outer membrane or construct surface may be expressed proportional to the dissolved oxygen concentration difference between the medium bulk and the outer construct surface through the solute transport coefficient k_c (i.e., the reciprocal external resistance to solute transport). The external mass transport resistance is often lumped in a thin stagnant liquid film adherent to the outer surface of the membrane/construct. Its actual value is generally

provided in non-dimensional semi-empirical equations correlating the Sherwood number $Sh=k_c\ d/D$ with powers of non-dimensional groups accounting for the geometry of the flow channel and the liquid velocity field (e.g., the Reynolds number $Re=\rho\ v\ d/\mu$), and the liquid and solute transport and physical properties (e.g., the Schmidt number $Sc=\mu/\rho\ D$, where μ is solvent viscosity, D solute diffusivity in the solvent, ρ solvent density), such as: $Sh \propto Re^{\beta}\ Sc^{\gamma}$ [35]. These correlations suggest that k_c is proportional to the β-th power of the liquid velocity relative to the construct v, and inversely proportional to the (1-β)-th power of the characteristic length of the flow channel or construct d, as follows: $k_c \propto v^{\beta} / d^{(1-\beta)}$. Hence, solute transport could be enhanced by increasing v and by decreasing d to an extent that depends on the value of β. The occurrence of turbulence and secondary flows promoted by mechanical stirring or tortuous flow around the membrane/construct generally yields higher β values and a stronger dependence of k_c on the liquid velocity than for liquid flowing in a tube under laminar conditions [36, 37].

In the analysis of external mass transport, it is often assumed that matter is ideally distributed outside the membrane/construct. The irregular and time-varying geometry of the construct, the geometry of the bioreactor inlet and outlet ports, improper construct fitting into the bioreactor and the actual operating conditions may all cause a non-ideal distribution of matter in the bioreactor which exposes the external surface of the construct to solute concentrations spatially varying in unpredictable fashion. As very nicely pointed out in [33], the occurrence of important back-mixing (e.g., caused by velocity gradients in planes orthogonal to the bioreactor or construct axis) may even off-set some of the advantages of external convective transport. The intensity of these non-ideal behaviors is often expressed in terms of a dispersion coefficient, or similar model parameters, accounting also for diffusion [38]. Although information is available in literature to estimate the dispersion coefficient for some industrial chemical reactors, its value and the occurrence of flow maldistribution in bioreactors for liver tissue engineering is more effectively estimated in the course of tracing experiments [39].
In a few bioreactors proposed for EC liver support, the patient's blood flows in direct contact with the membranes protecting the liver cell-seeded constructs [40, 41]. The use of whole blood significantly enhances oxygen external transport to the liver cell construct. In fact, the arterial blood of the average healthy individual may carry ca. 8.6 $mmol_{oxygen}/L_{blood}$ for the oxygen binding capacity of hemoglobin in the red blood cells, an amount far higher than that of aqueous medium [42]. However, in these bioreactors the enhancement of transport by

convection is limited by the possible occurrence of hemolysis or clotting caused by steep velocity gradients or blood stagnation in the bioreactor.

Membrane transport – Semi-permeable membranes are effectively used as immune barriers to protect xeno- or allogeneic liver cells in a construct implanted *in vivo* or used for EC liver support from immuno-competent proteins and cells [43]. Their presence causes an additional resistance to mass transport. Depending on their charge, size and physical-chemical properties (e.g., hydrophobicity) relative to that of the membranes, soluble metabolites may partition between the medium/blood and the membrane ending up with concentrations in the membrane at times significantly different than those in the medium. Solutes may be transported across the membrane by both diffusion and convection in response to concentration or pressure gradients across the membrane wall. The rate at which they are transported is effectively expressed as a function of the solute concentrations in the medium near the membrane face C_B and in the cell compartment near the membrane face C_C according to the irreversible thermodynamics as follows [44]:

1. $$J_S = P_M (C_B - C_C) + (1-\sigma) J_v \overline{C}$$

with $$\overline{C} = \frac{C_B - C_C}{\mathrm{Ln}(C_B / C_C)} \quad .$$

A flat membrane resistance to solute diffusive transport R_M may be expressed in terms of the reciprocal membrane diffusive permeability towards the given solute P_M, with:

2. $$R_M = \frac{1}{P_M} = \frac{\delta}{K\ D_M}$$

where δ is the membrane wall thickness, D_M the effective solute diffusivity in the membrane, and $K = C_{BM} / C_B = C_{MC} / C_C$ the solute partition coefficient in the case that the solute partitions equally between the medium and the membrane, where its concentration is C_{BM}, and the cells and the membrane, where its concentration is C_{MC}. The radial change of membrane surface area is also generally accounted for in R_M in the case of thick cylindrical membranes. D_M was shown to decrease with increasing solute-to-pore size ratios and with the solute molecular weight more rapidly than that predicted by the Einstein-Stokes equation [45]. The solute ability to be freely dragged across the membrane is expressed in terms of σ, the Stavermann coefficient, which is 0 for fully permeable and 1 for fully rejected solute. The medium flow through the membrane $J_v = L_p\ \Delta P$ is related to the membrane hydraulic permeability L_p and the pressure difference that develops, or is applied, across the membrane ΔP. The accumulation and

adsorption of partially or fully rejected solutes at the membrane surface may significantly change the membrane transport properties and decrease its permeability to both aqueous medium and soluble metabolites, in particular those of medium to high molecular weight [46].

Internal transport – Transport inside 3D cell constructs of soluble metabolites that are consumed or produced by the cells occurs simultaneously to their metabolic consumption or production. Under steady conditions, the metabolite concentration profiles that establish across the construct balance out the rate of physical transport into the construct with the rate of its consumption or production. In densely packed cell constructs, and in many bioreactors proposed in BALs for EC liver assist, soluble species are mainly transported by diffusion into the construct. This is particularly true for small solutes. Both the increasingly high cell density as cells grow and the amount of synthetic or newly formed ECM decrease solute mobility in the construct $D_{eff} \approx D\ \varepsilon/\tau$ as compared to that in medium D by decreasing its void fraction ε and by increasing its tortuosity τ. For purely diffusive transport, reduced solute diffusivity, high cell packing density and metabolic activity are all expected to contribute to a sharp decrease of soluble nutrient concentration into the construct away from the nutrient source. A more quantitative description of phenomena occurring in the construct may be provided according to a pseudo-homogeneous approach to predict the solute concentration profile across liver cell constructs formed inside or outside hollow fiber membranes [47-49]. As an example, let us consider the simple case of steady oxygen transport across a cell-seeded construct shaped as a slab and zero-th order oxygen consumption kinetics. The one-dimensional mass balance equation for oxygen may be written in terms of its non-dimensional concentration $C_{O2}/C_{O2,C}$ and the spatial coordinate x/L as follows:

3. $$\frac{d^2(C_{O2}/C_{O2,C})}{d(x/L)^2} = \frac{k_{oxy}\,C_{cell}}{D_{eff,O2}\,C_{O2,C}}\,L^2 = \phi^2$$

Equation 3 suggests that steep non-dimensional oxygen concentration profiles establish across the slab at high cell packing density C_{cell}, oxygen consumption rate on a per cell basis k_{oxy}, and slab half thickness L, and at low oxygen effective diffusivity in the construct $D_{eff,O2}$ and dissolved oxygen concentration at the outer slab surface $C_{O2,C}$ (equal to that in the medium bulk $C_{O2,b}$ for negligible external and membrane mass transport resistance). The actual oxygen concentration profile is determined by these variables grouped into the non-dimensional Thiele modulus ϕ rather than independently, values of $\phi^2>1$ causing a steep oxygen concentration decay into the construct. For $C_{cell}=10^8$ cell/mL, diffusivity in the cell mass $D_{eff,O2}=0.6\ D_{O2}$, k_{oxy},

K_m and $C_{O2,C}=C_{eq}$ as reported above, integration of Equation 3 yields at the center of a slab 200μm thick a dissolved oxygen concentration only 16% of that at the external face, and that anoxic conditions establish already at ca. 70μm from the external face of a slab 240μm thick. In the presence of external resistance to mass transport, the concentration of metabolic nutrients at the external slab face is lower than that in the medium bulk (it is higher for metabolic products) further reducing its average value within the construct. Under these conditions, the dimensional analysis of Equation 3 and its new boundary conditions suggests that the actual solute concentration profile across the construct depends also on the Biot number *Bi,* where $Bi=k_c\ L/D_{eff}$, and k_c is the solute transport coefficient that accounts for the serial transport external to the construct and across the membrane. The balance of external transport and metabolic consumption at the external slab surface suggests that the actual nutrient concentration at the external face is better determined by the Thiele-to-Biot number ratio ϕ^2/Bi [38]. The above analysis evidences that soluble nutrients transported only by diffusion may be supplied to the cells located in the center of only relatively small constructs or cell clumps and prevent their necrosis. Similar considerations apply also to proteic effectors (e.g., EGF) or toxins externally supplied to the cells in the construct. Steep effector concentration profiles across the construct might cause heterogeneous responses of cells at varying positions in the construct during new tissue formation. Steep toxin concentration profiles might cause the inefficient use of the cell detoxification activity, because only cells in the outer construct layers would contribute to the elimination of blood/plasma-borne toxins in EC liver support.

Pressure-driven perfusion of the construct adds some convection and enhances internal transport particularly of large, slowly diffusing molecules. Medium or plasma convective transport through liver cell clusters cultured around and in between hollow fiber membranes has been promoted by Starling flows [40,50] or by directly applying a pressure difference [51,52]. Convection-enhanced oxygen and nutrient transport was reported to maintain primary porcine and human cells viable and functional in a differentiated state for up to a few months. However, its effect on cell re-organization and new liver tissue formation depends on the balance of nutrients and waste metabolite transport to and away from the cells, respectively, and the retention of newly synthesized ECM components as liver-like tissue forms and its hydraulic resistance increases [31,53,54]. Pressure-driven medium or plasma perfusion of highly packed aggregates of different liver cell types co-cultured outside a 3D membrane network was even reported to promote the formation of sinusoid-like *canaliculi* among

aggregates of parenchymal cells [55,56]. When medium flows in the voids (also termed pores) around and among cell clumps, so dense that soluble species mainly diffuse to/from the cells into the clump, internal mass transport lends itself to a similar analysis as that described at the beginning of this paragraph. In each pore into the construct, the relative importance of convective and diffusive transport along the pore axis may be estimated in terms of the non-dimensional Peclet number $Pe=v\ d/D_{eff}$, where d and v are the pore size and liquid velocity into that pore respectively.

Cell metabolism – Cell sourcing for liver TE has been the subject of controversial discussions and remains a topic of major importance. The metabolic functions that a TE liver construct may have to provide are often limited to those typical of the hepatocytes, but might encompass all those performed by the liver as a whole. Primary liver cells may be isolated from mammalian tissue (as adult or stem cells) or may be genetically engineered (to proliferate quickly and be available off-the-shelf in large amounts). Primary cells may derive from the patient (i.e., autologous cells, including adult stem cells), from human donor tissue (i.e., homologous or allogeneic cells, including embryonic stem cells), or from an animal source (i.e., xenogeneic cells). The ideal source should provide cells functionally equivalent to human liver cells, with unlimited (but controllable) expansion capability, and should expose the patient to minimal risks of either an immunogenic response or transfer of infections. Cells should withstand the toxicity of the blood of ALF patients and should not lead to the liver construct premature failure. An important safety issue with important consequences on the TE construct function is the immunogenic response elicited by the cells. In traditional organ transplantation practice, immunogenic responses are categorized as either graft-vs.-host or host-vs.-graft. Depending on how the construct contacts the tissues and fluids of the host, these responses may be primarily mediated either by cells or by humoral factors. The characteristics of the primary mediator shall dictate the barrier properties of the membranes that may be used to prevent the construct rejection but may also affect the transport of other species, in particular large proteins [43].

The level of cell differentiation affects the liver-specific metabolic functions expressed and the cell proliferation capability. Along the differentiation spectrum, early embryonic cells are the prototypical undifferentiated cells that may become any cell type in the body (totipotency) and possess remarkable proliferative potential. As differentiation progresses, cells lose these two characteristics till they become terminally differentiated and express the whole repertoire of liver-specific functions but with little, if any, proliferative potential.

Adult primary hepatocytes are terminally differentiated and may express the full range of hepatocyte-specific functions in the proper *in vitro* culture environment. In fact, hepatocytes rapidly lose the liver-specific gene expression and become phenotypically unstable following isolation [57]. Initial clinical studies employed primary adult porcine liver cells for extracorporeal liver support [50,57]. Their use is controversial for various reasons, including the possible transfer of porcine endogenous retroviruses and possible immunologic reactions elicited by newly synthesized xenogeneic proteins [59]. Moreover, although the liver functions of different mammals are remarkably similar, differences do exist. A multivariate analysis of variance of patients treated with extracorporeal liver perfusion from 1964 to 2001 showed that only baboon and human livers provide an independent positive prognostic marker for improved survival [60]. This analysis suggests that xenogeneic hepatocytes are not the ideal substitute for the complex metabolic tasks of a human liver. Primary human liver cells isolated from explanted organs unsuitable for OLTx are an interesting cell source as they are ethically acceptable and capable of performing the human liver metabolic and regulatory tasks. According to the data of Eurotransplant and the European and American organ procurement organizations, approximately 20-25% of all explanted livers are unsuitable for transplantation and are discarded. Their number corresponds to that of the ALF patients that should be bridged to OLTx [61]. However, the viability of cells from these organs immediately after isolation may be impaired by suboptimal organ preservation and cell isolation procedures. It is worthwhile recalling that the survival, growth and metabolic functions of adult primary liver cells cultured *in vitro* in a scaffold are also affected by the culture technique, the medium composition, the dissolved oxygen tension, and the scaffold geometry and surface properties. Various immortalized cell lines have been developed that express liver-specific functions [17,18]. They are available off-the-shelf, have less stringent metabolic requirements than adult liver cells, and proliferate fast. However, they perform only some of the hepatocyte metabolic functions [62]. Concerns also exist for the oncogenic potential of cell lines derived from tumour cells.

Above it was discussed that the rate at which cellular metabolic reactions occur anywhere in the construct has important effects on the concentration profiles and gradients of soluble nutrients, metabolic wastes, toxins and proteic effectors across liver cell-seeded constructs. Reliable quantitative predictions of the relevant metabolic reaction rates for cells cultured according to different techniques, under different metabolite concentrations (i.e., their kinetics or rate equation) and at varying stages of liver tissue formation and maturation is essential to

make liver cells in the construct survive, migrate, differentiate, function and re-arrange in liver-like hierarchical structures [13,34,63]. Nonetheless, only a few rate equations are currently available for liver cell metabolic reactions. This is partly due to the fact that, currently, there is no clear definition of the functions that need to be replaced in EC liver support, nor of the minimal metabolic requirements [64]. Recent research has focused mainly on the quantitative characterization of the cell oxygen consumption kinetics. In fact, the dissolved oxygen concentration at the cell surface was shown to limit liver cell survival and function [26,28,29] and to control the enzymatic activities expressed by the liver cells [65]. The kinetics of cell metabolic reactions was mostly expressed independent of metabolites concentration (i.e., as zero-th order kinetics) and often estimated under largely uncontrolled environmental conditions. A few reliable equations correlate the cell oxygen consumption rate (OCR) to the dissolved oxygen concentration at the surface of primary porcine or rat hepatocytes immediately, or shortly, after seeding on planar scaffolds [28,29]. Some rate equations are available that correlate the ammonia elimination rate, urea synthesis rate and oxygen consumption rate of primary rat liver cells with the ammonia concentration at the cell surface in the post-attachment phase [66]. However, liver cells do not always respire at basal rates. In fact, challenging rat liver cells with increasing ammonia concentrations was reported to increase OCR [67]. Several factors were qualitatively shown to affect liver cell metabolism. The dissolved oxygen concentration and medium composition were reported to affect the enzymatic activities expressed by the liver cells [26,68]. Liver cells cultured on scaffolds differing for geometry (i.e., 2D vs. 3D), chemical composition and morphology were reported to synthesize proteins and to eliminate ammonia to a different extent [69-71]. High lactate and ammonia concentrations were reported to be toxic for liver cells to an extent depending on the exposure time [27]. In the co-culture of different liver cell types, cells with different oxygen metabolic requirements might also compete for the available dissolved oxygen. In spite of its importance, quantitative information on these effects is still lacking and complicates the optimization of bioreactor design and operation for the culture of liver cell constructs.

Bioreactors proposed for liver tissue engineering

Bioreactors for cell seeding

Cells are generally seeded on/in scaffolds in batch bioreactors that are sometimes also used for cell culture and for promoting tissue formation. They may be classified as static or dynamic,

depending on whether cell transport towards and into the scaffolds occurs by gravity or is promoted by a net mass flow across the scaffold, respectively.

Static bioreactors – Liver cells are still mostly manually spread on 2D or 3D scaffolds and let settle in stagnant medium (i.e., static seeding), with an efficacy strongly dependent on the operator's skills. Gravity or capillary forces promote cell transport from the suspension bulk to the outer scaffold surface and into its pores. It is well known that the initial cell density on 2D scaffolds has to exceed a critical value for cells to form monolayers. Below such critical value, liver cells adhere as singlets or doublets on biomaterials presenting adhesion peptide sequences. On very hydrophobic biomaterials, cells preferentially interact with one another and re-organize in clusters [72,73]. For its simplicity, static seeding has been and still is used for distributing liver and other cells (as such or in clusters) on/into different types of small and large 3D porous scaffolds [40,50,74,75]. However, static operation was reported to result in low seeding efficiency and cell density rapidly decreasing from the outer to the innermost zones of the scaffold [76,77].

Dynamic bioreactors – A few batch bioreactors have been proposed for the dynamic seeding of cells in scaffolds a few mm's thick and large. Slightly higher seeding efficiency and uniformity than in static bioreactors were reported by seeding chondrocytes and fibroblasts into porous scaffolds in stirred bioreactors (i.e., spinner flasks) [78,79]. In spinner flasks, the scaffolds are held in stationary positions in the medium and cells are dragged towards the scaffolds by the relative motion of the cell suspension promoted by mechanically or magnetically driven stirrers. With liver and other cell types and scaffolds, the cell seeding efficiency was not always reported to be higher than in static bioreactors [76,77]. In fact, the unidirectional medium flow causes cells to adhere more densely along the leading edge of the scaffold directly exposed to the suspension flow than in its trailing part. Other bioreactors exploit convection promoted by direct perfusion of the liver cell suspension through the scaffold driven by pressure gradients applied across the scaffold [52] or by centrifugal forces [21]. Independent of the cell type, direct perfusion of the scaffold pores enables the seeding of concentrated cell suspensions, and yields higher seeding efficiency and more uniform cell distribution throughout the scaffold as compared to seeding under static conditions or in spinner flasks [77,80]. However, long-term scaffold colonization requires high initial cell densities [21].

The size and structure of clinical-scale TE liver constructs used in BALs limits the use of dynamic seeding techniques. However, some degree of mixing is often generated after statically seeding the cells to improve the uniformity of the distribution and cell penetration in 3D porous scaffolds. Good liver cell penetration and distribution was reported in a 3D network of different semi-permeable hollow fiber membranes by static seeding a concentrated cell suspension (i.e., ca. $6x10^{10}$ cells/ml) followed by slow rotation of the bioreactor for the next 8 hours in the absence of direct perfusion [55,81].

A rather controlled sparse distribution of hepatocytes was obtained inside semipermeable hollow fiber membranes by injecting in the membrane lumen a viscous cell suspension in collagen and by letting it gel at 37°C. As the collagen gel forms, it blocks the cells in spatially ordered positions [82].

Bioreactors for tissue culture

Immediately after isolation or thawing, primary or immortalized liver cells are often seeded and cultured directly in the bioreactors where tissue should form. Only when used for investigating their metabolism, primary liver cells are shortly cultured under static conditions to make them recover from the isolation and purification stress. Most bioreactors proposed for liver tissue culture have been designed and operated so as to optimize the transport of nutrients to and waste metabolites away from cells densely packed into the construct, to enhance cell-cell contact and communication and the formation of neo-sinusoids to foster cell zonation, to avoid cell necrosis at the center of the construct, and to promote the expression of similar enzymatic activities as in the natural liver. Most bioreactors are dynamic because pressure-driven convective transport is promoted to minimize the mass transport resistance external and internal to the construct, and across the membranes when they are used. The requirements that the bioreactors should fulfil change with the application and have led to a great variety of bioreactors differing for design, operation mode, cell culture technique and scaffolding [47,83]. In the following, the bioreactors proposed for the BALs used for the EC liver support of ALF patients, for growing implantable TE liver constructs, and for investigating liver cell metabolism or for toxicological drug screening are presented.

Bioreactors for BALs

EC liver support aims at maintaining ALF patients in a stable neurological status till a tissue-compatible liver is available for OLTx or until the own liver heals. In BALs, liver cell

constructs are cultured in bioreactors to provide ALF patients with the biosynthetic, regulatory and detoxification capacity lacking to their diseased liver and necessary for stopping progression of the damages of liver failure or promoting healing of the liver parenchyma. At least in principle, the bioreactor is required to perform only a subset of all the functions of the natural liver, to an extent equivalent to roughly one third of the natural liver cell mass, consistently for a time ranging from a few days to a few weeks. Moreover, the bioreactor should feature a low priming volume, be ready and easy to use in the clinical setting, and should not be hazardous to the patient or too expensive. In some BALs, passive membrane-based or adsorptive blood/plasma detoxification devices provide for the needed detoxification capacity or augment that of the bioreactor, as shown in Figure 1 [50,84].

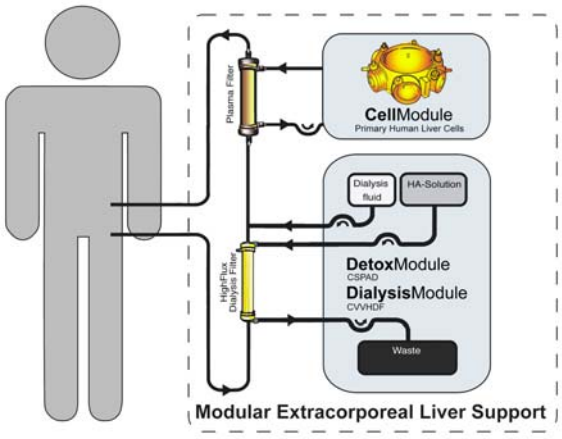

Fig.1. Scheme of the Modular Extracorporeal Liver Support (MELS) BAL equipped with the *Cellbioreactor*Module bioreactor module, the *Detox*Module for toxin removal by dialysis against a concentrated human albumin (HA) solution, and the *Dialysis*Module for renal support.

In the early bioreactors, liver cells were cultured in the shell of commercial, off-the-shelf dialysis, filtration or plasmapheresis hollow fiber membrane modules as cell suspensions, bound to microcarriers or in adhesion on the outer membrane surface as shown in Figure 2a [40,50,85].

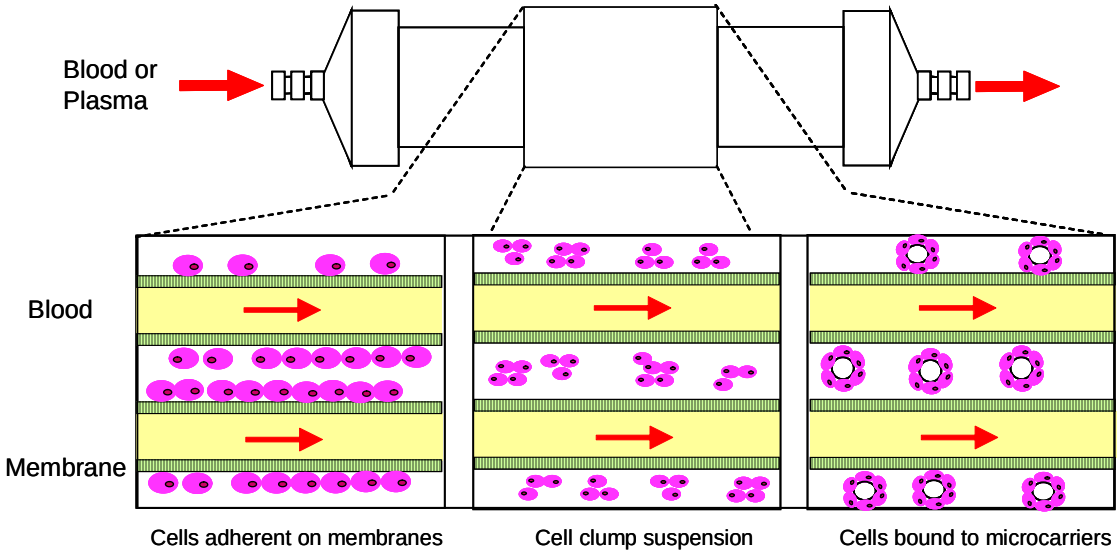

Fig. 2A. Scheme of the bioreactors proposed for BALs used for extracorporeal liver assist and tested in the clinics: a) liver cells cultured outside hollow fiber membranes in shell-and-tube configuration as a suspension of cell clumps, bound to microcarriers, or in adhesion on membrane surface, with blood, plasma or medium flowing in the membrane lumina.

Starting in the mid 90's, a new generation of bioreactors became available in which liver cells were cultured in 3D scaffolds (e.g., in a gel bed, non-woven fabrics, foams, or a membrane network) that were to replace the natural ECM (Table 1), and oxygen supply to the cells was enhanced by inclusion in the construct of distributed oxygen sources, as schematically shown in Figures 2 c-e [51,75]. Direct construct perfusion with medium, plasma or whole blood was also exploited to minimize the external or internal mass transport resistance, or both (Figures 2 b-e). Most of the proposed bioreactors are operated in recycle mode to minimize transport resistance and maximize the yield of liver cell reactions. Some of the proposed laboratory-scale continuous-flow bioreactors were tested *in vitro* (Table 2), and were rapidly scaled-up to treat small-to-large animal models of ALF (Table 3). Only a few were scaled-up to treat ALF patients and are under clinical assessment (Table 4 and Figure 2).

Table 1. *In vitro* liver cell culture techniques

Culture technique	Cell Type	References
Suspension of single or aggregated cells	Rat hepatocytes	86
Cell entrapment in microcapsules	Rat hepatocytes or Chang liver cell line	87,88,89
Cell aggregates adherent on beads	Porcine hepatocytes	50
Cells adherent on the external surface of parallel hollow fiber membranes	Human hepatoblastoma C3A cell line	40
Cells embedded in a collagen gel	Rat or human hepatocytes	2, 90
Cells outside and in between hollow fiber membranes orderly organized in a 3D network	Porcine or human liver cells	51,91
Cells in 3D foams	Porcine hepatocytes	92,93
Cells in 3D non-woven fabrics	Porcine hepatocytes	75,94
Cells in porous biodegradable polymeric scaffolds prepared by 3D microprinting	Rat hepatocytes	95
Cells adherent on borosilicate wafers with micropatterned biochemical cues	Rat hepatocytes and 3T3 fibroblasts	96

Bioreactors where liver cells are cultured outside hollow fiber membranes arranged in a shell-and-tube configuration in suspension, as cell clumps, or bound to microcarriers have been the first ones proposed for BALs (Figure 2a). They have a number of interesting features: cell seeding is easy, quick and can be done on demand by using cryopreserved tissue [50]; the size of cell aggregates is not limited by the membrane diameter; cell grafts need not to be vascularized; membranes protect the allografts against rejection; commercial membrane modules may be used. However, in bioreactors equipped with hemodialysis or low flux hemofiltration membranes the liquid in the cell compartment is generally stagnant and causes a large resistance to metabolite transport to/from the cells possibly leading to cell starvation and the accumulation of waste metabolites. In recent commercial versions of these bioreactors mixing in the shell-side is increased by changing their aspect ratio and by using more permeable membranes so as to promote the occurrence of Starling flows. Concern exists for cell sedimentation in use, and for cell viability and capacity to express liver-specific metabolic functions in the long term in particular when cell suspensions are used. Bioreactors of this type, seeded with cryopreserved porcine liver cells attached to dextran microbeads, have been used

in a BAL shortly after cell seeding to treat the plasma of ALF patients for up to 4-6 h at a time [113].

In another early bioreactor concept for BALs, cells of the immortalized C3A line are cultured in adhesion on the external surface of hollow fiber (HF) membranes arranged in an acrylic housing in shell-and-tube configuration while blood or plasma flows in the membrane lumina. Sussmann *et al*. reported that bioreactors of this type containing ca. 200g of cells in the shell reversed ALF in a dog model [40], permitted ALF patients to regain consciousness and promoted liver regeneration [112]. Later on, Millis *et al*. reported similar results treating the plasma of ALF patients in EC circulation with 4 of these bioreactors in parallel [119]. Culturing liver cells outside and among HF membranes permits cell 3D organization and the culture of a large cell mass. The metabolic requirements of immortalized cells derived from tumour cells are also less important than those of primary cells. However, the dense cell packing in the shell and the low hydraulic permeability of the hemodialysis membranes used permit transport of soluble metabolites to/from the cells only by diffusion. This may cause steep concentration gradients in the cell mass and may lead to depletion of essential nutrients and cell necrosis in the regions farther away from the membrane surface, as has been reported. Since tumour-derived liver cells are used, a concern is the potential release of soluble carcinogenic species or the leak of cells into the patient's circulation. For this reason, Millis *et al*. filtered the treated plasma across microfiltration membranes prior to returning it to the patient.

Liver cell inclusion in a homogeneous collagen or alginate gel bed provides a large surface area for 3D cell scaffolding and was reported to promote cell polarization, differentiation and cell-cell contacts, and to yield viability and differentiated functions higher than monolayer culture and stable for weeks. It may also permit a good control over cell distribution across the bed depth and along its length. Nyberg *et al*. [82] used primary liver cells embedded in a collagen gel inside the bore of hollow fiber semi-permeable membranes assembled in a shell-and-tube configuration for the EC treatment of the blood of ALF patients flowing outside the membranes (Figure 2b). Blood flow around the membranes decreases mass transport resistance external to the membranes that protect the allograft against rejection. However, suboptimal design of the blood flow paths may cause blood clotting. Cell necrosis at the innermost regions of the bed has also been reported possibly caused by the steep nutrient and oxygen concentration gradients that develop across the bed depth.

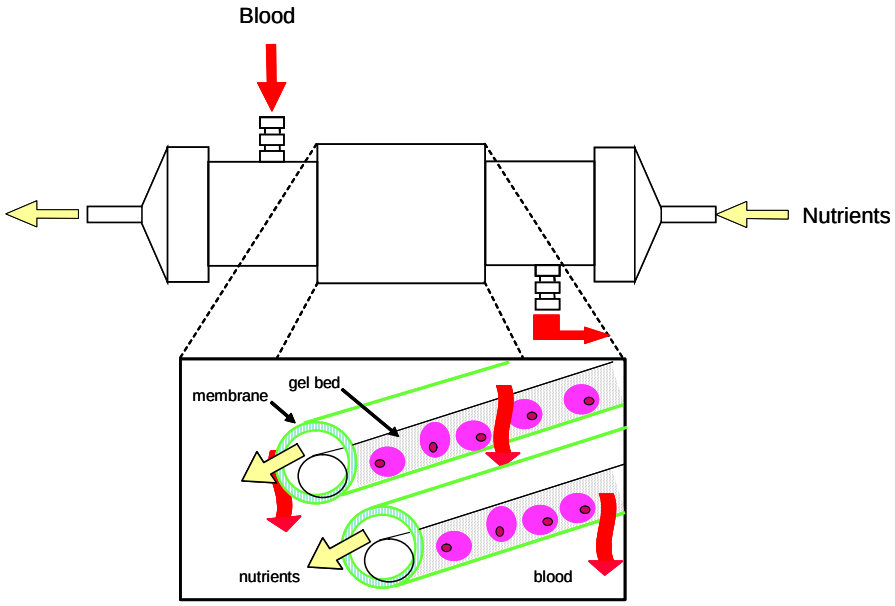

Fig. 2b. Liver cells embedded in a collagen gel inside the lumen of hollow fiber membranes in shell-and-tube configuration with blood or medium flowing outside and along the membranes;

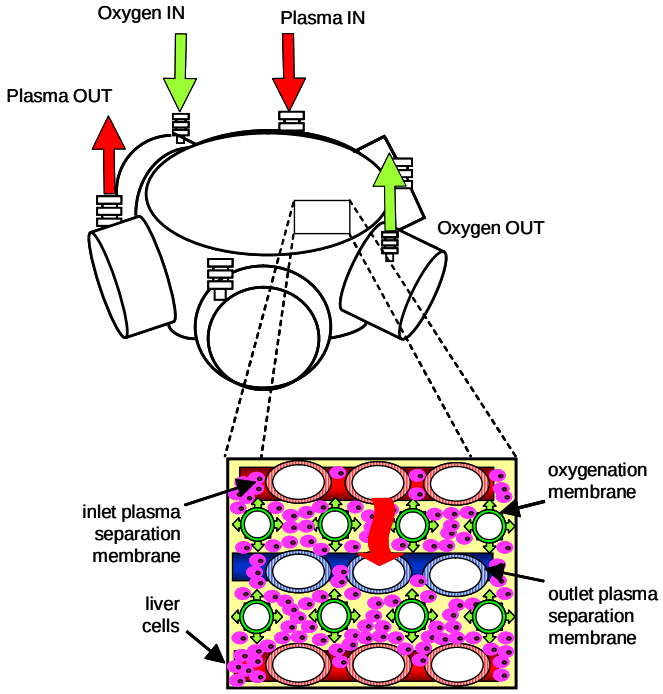

Fig. 2c. Liver cells cultured outside and among different hollow fiber membranes orderly organized in a 3D network with a distributed oxygen supply, with plasma or medium filtering from one membrane bundle to the next through the cells (see text).

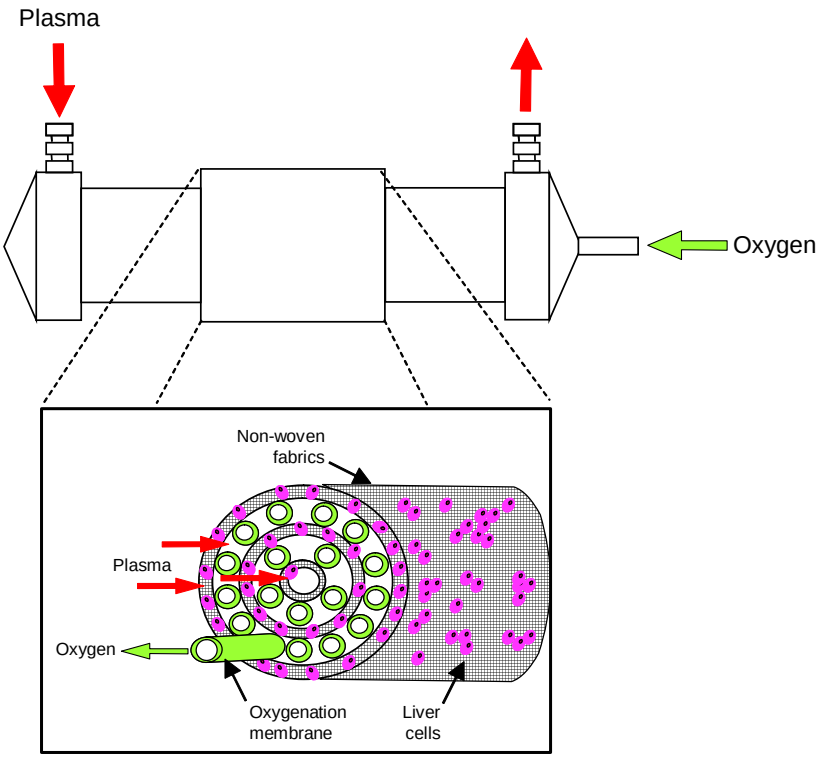

Fig. 2d. Liver cells attached to a 3D fabric with a distributed oxygen supply, with plasma or medium axially flowing along the fabric.

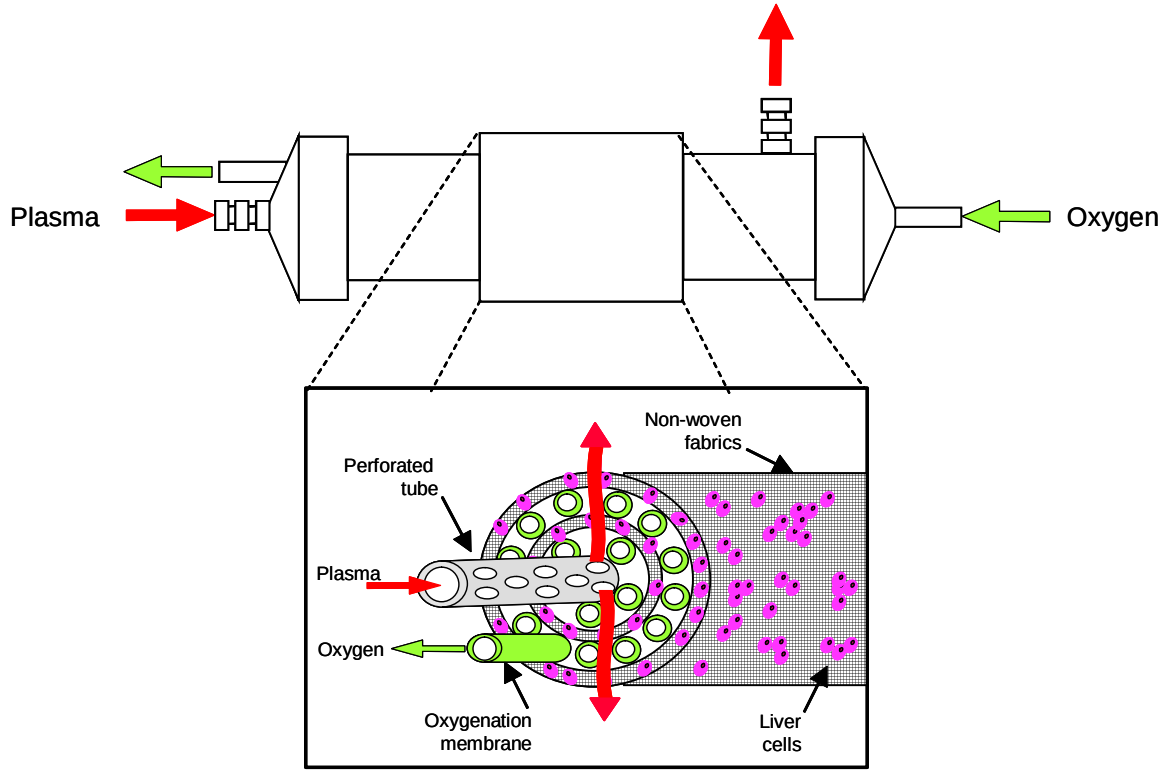

Fig. 2e. Liver cells attached to a 3D fabric with a distributed oxygen supply, with plasma or medium radially flowing across the fabric.

A four compartment bioreactor based on a network of interwoven hollow fiber membranes was proposed for liver cell culture by Gerlach *et al*. [51,91] (Figure 2c). Aiming at reproducing the liver vascular network, the bioreactor consists of a 3D network of HF membranes with different separation and transport properties orderly woven in planar mats enclosed in a polyurethane housing. The repeating basic network unit consists of two mats of hydrophilic microporous HF membranes, with the membranes in a mat angled at 90 degrees with respect to those in the underlying mat, in between which a mat of hydrophobic microporous HF membranes is interposed for oxygen supply to and CO_2 removal from the cells. The membranes in mats with the same properties and orientation are bundled up together and fitted with headers. Porcine or human primary parenchymal and non-parenchymal liver cells are co-cultured outside and among the membranes in the network. Medium or plasma is fed to the bore of one bundle of hydrophilic membranes, a fraction (i.e., cross-flow operation) or the whole stream (dead-end operation) filters across the membranes, bathes the cells, contacts the oxygenation membranes and is replenished with oxygen, is re-absorbed in the bore of the hydrophilic membranes in the other bundle, and is returned to the patient's circulation or the accumulation vessel. Up to two more bundles of different membrane mats may be added to the network to introduce additional functions (e.g., to augment gas exchange, dialyze out small solutes, feed nutrients etc.). In this bioreactor, oxygen is supplied to the cells with the medium but is also locally supplied through the hydrophobic microporous membranes causing more physiological oxygen gradients to establish across the cell mass. Pressure-driven direct cell perfusion enhances transport of large solutes and species rapidly consumed/produced to/from the cells and should lead to the prompt return of large liver-specific factors to the plasma, reduced accumulation of waste metabolites near the cells, enhanced cell survival and functions, and the efficient use of the available cellular activity. Indeed, liver cells cultured in the 3D membrane network were shown to spontaneously re-organize in liver-like aggregates forming sinusoid-like microchannels with a neo-space of Disse. Cells produced biomatrix and expressed liver-specific functions consistently for several weeks. BALs using this bioreactor for the EC treatment of pig models of ALF gave encouraging results [107,108]. Bioreactors seeded with porcine liver cells were used in BALs as a bridge to OLTx to treat ALF patients (Figure 3), coma stage III-IV, leading to a 100% survival rate after 3 years from treatment and OLTx [116]. A pilot study aimed at using the same bioreactor in the treatment of ALF patients but seeded with human liver cells harvested from donor organs discarded for steatosis, cirrhosis or mechanical injury is currently underway and is giving promising results [117].

Fig. 3. Clinical treatment of an ALF patient with the Modular Extracorporeal Liver Support (MELS) BAL utilizing the bioreactor developed by Gerlach *et al*. [51] loaded with 600 g of primary porcine cells.

In 1997, Flendrig *et al*. proposed another packed-bed bioreactor with decentralized oxygen supply permitting direct perfusion of high density liver cells with low nutrients concentration gradients [75]. Primary porcine liver cells were cultured attached to the fibers of a spiral wound 3D polyester non-woven fabric packed in a cylindrical acrylic enclosure, and were directly perfused with medium or plasma flowing along the bioreactor length (Figure 2d). Microporous membranes interposed in between adjacent fabric layers provided for a distributed oxygen supply and CO_2 removal. Hepatocytes were reported to arrange in the fabric in *in vivo*-like aggregates, to synthesize urea and proteins, and to transform lidocaine into MEGX and xilidine for up to 2 weeks. Use of the bioreactor for the EC treatment of animal models of ALF caused a significant enhancement of the survival rate of small and large laboratory animals [109] and was proven safe in the treatment of ALF patients [118]. Recently, Ambrosino *et al*. proposed to couple the polyester fabric with a porcine autologous biomatrix to enhance cell attachment to the 3D scaffold [99]. In 1996, Naruse *et al*. [94] and later on Morsiani *et al*. [103] modified this concept by arranging the fabric in an annular packed-bed bioreactor and by flowing medium or plasma radially across the fabric to enhance oxygen transport to the cells and reduce the bioreactor inlet/outlet pressure drop (Figure 2e).

Up to ca. 230 g primary hepatocytes could be cultured in such bioreactor in a high metabolically active state [120]. BALs based on this bioreactor are under clinical testing.

Table 2. Continuous-flow bioreactors used for the *in vitro* culture of liver cell constructs.

Bioreactor type	Cell type	References
Hollow fiber membrane bioreactor in shell-and-tube configuration with cells adherent on the external membrane surface and medium fed to membrane lumen	Human hepatocytes or hepatoblastoma C3A cell line	40,97
Cylindrical bioreactor with rotating disks containing hepatocytes entrapped in a hydrogel with blood flowing at the bottom of the cylinder	Rat hepatocytes	98
Bioreactor with an inner 3D network of hollow fiber membranes with cells cultured outside and between the membranes, distributed oxygenation through one or more membrane bundles, and medium filtered across two membrane bundles to perfuse the cells	Co-culture of porcine or human liver cells	50,91
Cylindrical bioreactor packed with a cell seeded spiral wound polyester or polyurethane non-woven fabric, also coupled to biomatrix, with interwoven hollow fiber membranes for oxygen supply, and medium axially perfusing the fabric	Porcine or rat hepatocytes	75,99
Packed bed bioreactor with cells cultured in a porous 3D scaffold perfused with medium	Rat liver cells	92,93
Bioreactor with medium flowing along an adherent cell monolayer with oxygen supplied through a flat membrane hanging on the flow channel	Porcine hepatocytes	100,101
Cells cultured in the annulus between two coaxial hollow fiber membranes with medium filtered across the cell compartment from the neighbouring compartments and oxygen supplied to the medium flowing outside the outermost membrane	Rat hepatocytes	102
Annular bioreactor packed with a cell seeded spiral wound polyester non-woven fabric with interwoven hollow fiber membranes for oxygen supply, and medium radially perfusing the fabric	Porcine hepatocytes	94,103
Encapsulated cells in fluidized bed bioreactor	Cells of the hepatoblastoma C3A line	104
Bioreactor packed with a construct prepared by ionically etching silicon wafers featuring microchannels perfused with medium and cells adherent on the channel wall	Rat hepatocytes	105

Table 3. Continuous-flow bioreactors used for the treatment of animal models of ALF.

Bioreactor type	Cell type	References
Hollow fiber membrane bioreactor in shell-and-tube configuration with cells cultured in the shell as clumps adherent on microbeads and medium fed to membrane lumen	Rat or porcine hepatocytes	106
Hollow fiber membrane bioreactor in shell-and-tube configuration with cells adherent on the external membrane surface and medium fed to membrane lumen	Human hepatoblastoma C3A cell line	40
Bioreactor with an inner 3D network whose repeating module consists of two overlaid hydrophilic microporous hollow fiber membrane mats and a mat of microporous hydrophobic membranes interposed among the other mats for oxygen supply. Cells cultured outside and among the membranes are perfused by the medium filtered from the first to the second hydrophilic membrane mat.	Porcine liver cells	107,108
Cylindrical bioreactor packed with a cell seeded spiral wound polyester non-woven fabric with interwoven hollow fiber membranes for oxygen supply, and medium axially perfusing the fabric	Porcine hepatocytes	109
Encapsulated cells in fixed bed bioreactor	Porcine hepatocytes	110
Cylindrical bioreactor packed with cell seeded polyurethane foam	Porcine hepatocytes	92,93
Bioreactor with plasma flowing along an adherent cell monolayer with oxygen supplied through a flat membrane	Porcine hepatocytes	101

Table 4. Continuous-flow bioreactors used for the clinical treatment of ALF patients

Bioreactor brand name & type	Additional detoxification treatment	Perfusate	Cell number & type	References
Cell and charcoal suspension in a housing perfused with blood	Activated charcoal suspension	Blood	$40x10^6$ porcine hepatocytes	111
Vital Therapies ELAD® Shell-and-tube HF membrane bioreactor with cells adherent on the external membrane surface and blood fed to membrane lumen		Blood	$2x10^{10}$ (ca. 200 g) cells of human hepatoblasto-ma C3A line	112 Fig. 2a
Arbios Systems HepatAssist® Shell-and-tube HF membrane bioreactor with cryopreserved cell clumps adherent on dextran microbeads in the shell and plasma fed to membrane lumen	Activated charcoal adsorbent cartridge	Plasma	$4\text{-}6x10^9$ (ca. 50 g) cryopreser-ved porcine hepatocytes	113,114 Fig. 2a
Excorp Medical BLSS® Shell-and-tube HF membrane bioreactor with cells in the membrane lumen embedded in collagen and blood flowing around the membranes in the shell		Blood	$8x10^9$ (ca. 100 g) porcine hepatocytes	115 Fig. 2b
MELS CellModule 3D membrane network bioreactor with repeating units consisting of two overlaid hydrophilic microporous HF membrane mats and a mat of microporous hydrophobic membranes interposed among them for oxygen supply. Cells cultured outside and among the membranes are perfused by the plasma filtered from the first to the second hydrophilic membrane mat	Dialysis and stripping of hydrophobic toxins with a concentrated albumin solution possible	Plasma	$2.2x10^{10}$ (ca. 500 g) porcine or human liver cells	116,117 Fig. 2c

AMC BAL Cylindrical packed bioreactor with cell-seeded spiral wound polyester non-woven fabric with HF membranes for oxygen supply, and plasma axially perfusing the fabric		Plasma	$2x10^9$ porcine cells	118 Fig. 2d
Vitagen ELAD® Shell-and-tube HF membrane bioreactor with cells adherent on the external membrane surface and plasma fed to membrane lumen		Plasma	ca. 4 x 200 g cells of human hepatoblasto-ma C3A line	119 Fig. 2a
RAnD BAL Annular packed bioreactor with cell seeded spiral wound polyester non-woven fabric with HF membranes for oxygen supply, and plasma radially perfusing the fabric	Bilirubin adsorption cartridge	Plasma	$2\text{-}2.3x10^{10}$ porcine hepatocytes	120 Fig. 2e

Bioreactors for implantable TE liver constructs

The therapeutic success of split liver transplantation suggests a possible role for implantable engineered liver constructs in the treatment of acute, chronic or AoC liver failure. To make its implantation and subsequent integration possible, the TE liver construct should exhibit as similar an architecture as possible to that of the natural liver and should be easily connected to the vascular network of the liver. Bioreactors may be used to provide liver cell types with the biochemical and mechanical cues that promote the organization of different liver cell types in the construct. Only a few bioreactors have been proposed thus far to this purpose that permit the culture of only rather small liver cell-seeded constructs.

In the rotating wall vessel (RWV) bioreactor, liver cells may be cultured in small 3D biodegradable scaffolds suspended in medium in the space between two concentric cylinders. The continuous rotation of the medium was proven to reduce the effect of gravity causing cells to distribute uniformly throughout biodegradable scaffold, establish tight cellular junctions, form bile duct-like structures and synthesize proteins for up to 60 days [121]. The laminar flow of the medium with respect to the construct, and the continuous oxygen supply through membranes placed either on a cylinder or a flat wall, was reported to effectively reduce the resistance to essential nutrients and waste transport external to the construct without subjecting

the cells to high shear. However, only TE constructs of limited size may be produced with the RWV bioreactor.

Starting from the good results reported with other cell types, Kim *et al*. proposed to culture in a cylindrical continuous-flow bioreactor rat hepatocytes attached to tubes a few millimeters thick and long prepared from non-woven sheets of a biodegradable polyglycolic acid [95]. Dynamic cell seeding permitted cells to distribute uniformly throughout the scaffold (see above). The good 3D scaffold accessibility, made possible by 250 μm large inner pores and ca. 96% porosity, and the optimization of the oxygenated feed flow rate were reported to enhance significantly the transport to/from the cells and to yield a stable albumin production for about a week of culture.

Bioreactors for metabolic studies

Knowledge of the rate at which metabolites or drugs are produced or consumed by the liver cells in a construct under given operating conditions (e.g., metabolite and proteic effector concentrations, medium composition, pH, etc.) is essential for preventing necrosis at the core of the construct and for designing bioreactors for therapeutically effective BALs, and is generally expressed in terms of the rate equations (i.e., the kinetics) of the metabolic reactions in which a given metabolite or drug participates [38]. Kinetic characterization of the liver cell metabolic reactions in the presence of known amounts of drugs or toxins may also provide information on drug clearance and cytotoxicity that are important for the development of new drugs. The ideal bioreactor for liver cell kinetic characterization should permit cell culture according to the given technique, should feature a fluid dynamics that enable a controlled distribution of metabolite concentrations at the cell surface and their accurate measurement or estimation. Its design and operation should minimize the resistance to metabolite transport to the cells and should easily permit to extract the reaction rate from the overall bioreactor performance, by separating kinetics from physical transport. Such bioreactors do not need to host large amounts of cells, which makes the resistance to soluble metabolite transport internal to the construct less critical than in large TE liver constructs. However, similarly to those considered thus far, these bioreactors should promote liver cell re-organization in liver-like structures and the expression of the same enzymatic activities as in the natural liver. In consideration of the multiple reaction steps leading to the elimination of a drug, the bioreactors for drug screening as a whole should also yield metabolite concentration profiles as close as possible to those *in vivo*.

Static bioreactors - The kinetics of liver cell metabolic reactions is traditionally characterized in static bioreactors, such as the Petri dishes. They are well established, inexpensive and easy to use. The rate of the investigated metabolic reaction is generally estimated as the ratio of the bulk metabolite concentration difference to the finite time elapsed after adding the metabolite to the medium, and is related to the initial bulk metabolite concentration. However, such an estimate is the compound effect of reaction events taking place at varying rates as the metabolites (or drugs) are consumed or produced and should not be related to the initial metabolite concentration [63]. Time varying cell number or metabolic activity, and the existence of spatial metabolite concentration gradients caused by poor mixing or resistance to metabolite transport may make these estimates even less reliable. The effect of mass transport resistance is particularly significant when cells are cultured in 3D scaffolds or in adhesion at high surface density under a thick layer of medium [47].

Dynamic bioreactors – The intrinsic kinetics (i.e., unaffected by mass transport) of liver cell metabolic reactions is conveniently characterized in continuous-flow bioreactors operated under time-invariant conditions. In these bioreactors, fresh medium containing nutrients and metabolites continuously enters the bioreactor, flows in contact with the cells and leaves the bioreactor carrying away spent medium and waste metabolites, similarly to that occurring in the natural liver. Many dynamic bioreactors have been proposed for the kinetic characterization of liver cells cultured on/in 2D/3D scaffolds. In most cases the distribution of matter (i.e., the actual metabolite or drug spatial concentration profiles) in the bioreactor is unknown, nor is it clear the extent to which the reaction rate estimated as the overall bioreactor performance is affected by the physical transport of metabolite or drug to/from the cells. When the distribution of matter in the bioreactor and the transport resistance to/from the cells is characterized, quantitative information on the rate of the investigated metabolic reactions can be gathered from the steady state mass balance about the bioreactor for a given metabolite or drug. Rotem *et al*. [28] and later Smith *et al*. [122] characterized the oxygen consumption rate of liver cells embedded in a flat collagen sandwich or in adhesion on collagen, respectively, at the bottom of a modified T-flask operated at steady state under a constant medium height. The kinetics of oxygen consumption was characterized under the assumption that medium flows as a plug above the cells and that oxygen in the gaseous phase above the cells is in equilibrium with the dissolved oxygen in the medium anywhere in the bioreactor. For non-gaseous metabolites, a macroscopic mass balance about the bioreactor for the metabolite does not provide a direct estimate of the rate at which a metabolite is consumed or produced for their concentration

continuously varies along the bioreactor length, as the reaction rate does [38]. This complicates the characterization of metabolic reactions with complex, non-linear kinetics. Kinetic studies can be conveniently performed with continuous-flow well mixed bioreactors where metabolites are uniformly distributed. In fact, everywhere in the bioreactor cells are exposed to the same metabolite, effector and drug concentration, and metabolic reactions occur everywhere at the same rate. Concentrations in the bioreactor equal those in the stream leaving the bioreactor, that are easily and reliably measured. Moreover, at steady state, the metabolite concentration difference in the streams entering and leaving the bioreactor provides a direct estimate of the investigated metabolic reaction rate. Gebhardt *et al*. [123] and Smith *et al*. [122] proposed a one-compartment and a two-compartment continuous-flow bioreactor, respectively, that should have prevented spatial metabolite concentration gradients. However, no evidence was provided that soluble metabolites indeed distribute uniformly in the bioreactor, nor that the effect of mass transport resistance to/from the cells is indeed negligible (i.e., that cells were operated under kinetic control). Catapano and De Bartolo proposed a well mixed continuous-flow, recycle bioreactor for the characterization of the metabolic reactions of primary rat liver cells in adhesion on collagen [66]. The bioreactor was designed to host planar supports that can be inserted in standard Petri dishes to let liver cells recover from the isolation stress prior to the kinetic characterization. A fraction of the stream leaving the bioreactor is continuously recycled back to the bioreactor inlet where it mixes with fresh medium. The recycle makes it possible to separate control of the mixing in the bioreactor from the hold-up time (i.e., the bioreactor volume-to-feed flow rate ratio) to minimize the shear cells are subject to and to maximize the metabolite concentration difference about the bioreactor. The bioreactor design and operation (i.e., bioreactor volume, design of the inlet and outlet ports, feed flow rate, recycle ratio, etc.) was optimized to culture liver cells at uniform metabolite concentrations and under negligible mass transport resistance. This bioreactor was successfully used to characterize the dependence of the rate of oxygen consumption, ammonia and phenolsulphonphtalein elimination on ammonia concentration in the medium. Dynamic bioreactors have also been proposed as an alternative to traditional methods in drug screening and cytotoxicity assessment. Recently, a flat plate continuous-flow bioreactor similar to that of Rotem *et al*. was proposed by Allen & Bathia [124] to impose steady physiological gradients of dissolved oxygen and nutrient concentrations over hepatocytes in adhesion on collagen, as an *in vitro* model of liver zonation. In this bioreactor, the high metabolic consumption rates cause metabolite or dissolved oxygen concentration gradients to form along the bioreactor similarly to that occurring internal to 3D liver constructs. The distribution of matter and the dissolved

oxygen gradients in the bioreactor were validated by comparing experimental dissolved oxygen concentrations in the stream leaving the bioreactor with predictions from a plug flow transport model. The Authors reported that the oxygen gradients contribute to an *in vivo*-like heterogeneous induction of enzymatic activities (e.g., CYP2B and CYP3A), modulate the enzymatic activities induced with drugs, and reproduce well *in vitro* the zonal toxicity of acetaminophen reported *in vivo* [125]. A scaled-down version of the dynamic 3D membrane network bioreactor developed by Gerlach *et al*. [51] for a BAL, seeded with porcine liver cells and batch-wise operated by filtering the medium across the cell compartment in dead-end mode, was also shown to promote cell re-arrangement in liver-like structures and yield MEGX concentration profiles similar to those found *in vivo* when challenged with physiological lidocaine concentrations [126]. The kinetic analysis yielded lidocaine clearance comparable to those reported in literature and evidenced the importance of accounting for the adsorption of hydrophobic drugs to materials when assessing the drug clearance of liver cell constructs in bioreactors. A micro-bioreactor fostering the formation of 3D liver tissue has been recently proposed for the assessment of drug safety and efficacy by Powers *et al.* [127]. The bioreactor core is a 3D scaffold produced by deep reactive ion etching of silicon wafers featuring through microchannels approx 300μm x 300μm wide with collagen-coated cell adhesive walls. Rat hepatocytes were reported to adhere to the channel walls and re-arrange into liver-like structures when the scaffold pores were perfused at flow rates at which the oxygen transport rate to the cells matches their estimated OCR. However, cells are still exposed to physiological shear stresses. Seeding cell spheroids rather than single cells was reported to maintain tissue-like architecture and viability for long times. The Authors report that in this micro-bioreactor the hepatocytes expressed at near *in vivo* levels many drug metabolizing enzymatic activities that are rapidly lost *in vitro* in many other bioreactor systems [128].

Concluding remarks

The large number of bioreactors proposed for liver tissue engineering testifies to the ingenuity of researchers operating in the field. However, cell response to soluble and immobilized biochemical cues is only now beginning to be unraveled. Quantitative information on liver cell metabolic reactions at each stage of cell maturation and the type and amount of biochemical *stimuli* needed to promote liver-like tissue formation *in vitro* still needs to be gathered. Much work is still to be done towards the understanding and the modeling of transport phenomena in the proposed bioreactors. Within this framework, continuous-flow bioreactors purposely

designed, seeded and operated shall contribute to establishing *in vitro* the proper environment to promote cell re-organization in 3D scaffolds in liver-like hierarchical structures and cell differentiation to a stable adult phenotype. This way, EC treatments based on these bioreactors shall be able to provide for the needed functions to support ALF patients until OLTx or the own liver tissue regenerates. New bioreactors shall also constitute the core of effective *in vitro* model systems to investigate liver cell metabolism and replace costly, at times also unreliable, animal tests in the assessment of drug clearance and toxicity.

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