Current development of bioreactors for extracorporeal bioartificial liver (Review)[a)]

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The research and development of extracorporeal bioartificial liver is gaining pace in recent years with the introduction of a myriad of optimally designed bioreactors with the ability to maintain long-term viability and liver-specific functions of hepatocytes. The design considerations for bioartificial liver are not trivial; it needs to consider factors such as the types of cell to be cultured in the bioreactor, the bioreactor configuration, the magnitude of fluid-induced shear stress, nutrients' supply, and wastes' removal, and other relevant issues before the bioreactor is ready for testing. This review discusses the exciting development of bioartificial liver devices, particularly the various types of cell used in current reactor designs, the state-of-the-art culturing and cryopreservation techniques, and the comparison among many today's bioreactor configurations. This review will also discuss in depth the importance of maintaining optimal mass transfer of nutrients and oxygen partial pressure in the bioreactor system. Finally, this review will discuss the commercially available bioreactors that are currently undergoing preclinical and clinical trials. © *2010 American Vacuum Society.* [DOI: 10.1116/1.3521520]

I. INTRODUCTION

Acute liver failure (ALF) and acute-on-chronic liver failure (ACLF) pose exceptionally high risk of mortality among the liver diseases. They always require intensive life support and subsequent organ transplantation. The pathogenesis of these diseases comprises different pathological pathways with the important ones being accumulation of ammonia, upregulated systemic inflammation, and reversible to irreversible hepatocellular damage.[1] Due to the scarcity of grafts for liver transplants, extracorporeal liver support systems are expected to play a major role in reducing complications of the liver failure, and sustaining liver functions if the waiting time for the actual liver transplant is prolonged. Although there has been more than 2 decades of exploration in this field, further enhancements in the current systems coupled with properly controlled clinical studies are still necessary for the success of this application.[2–4]

Two types of extracorporeal liver support systems are available; they are the artificial and the bioartificial liver. Artificial livers are widely used in medical intensive care units. The therapeutic purpose of artificial livers is the removal of accumulated toxins in the body through their separation and absorption in the hollow fiber membrane. Although artificial liver is more commonly used in clinical setting than its bioartificial counterpart, it still faces several challenges pertaining to its clinical outcome and the validation of its therapeutic guideline.[5]

The idea of bioartificial liver is conceived based on the understanding that detoxification alone is not sufficient to replace the liver functions. It is well known that the liver is indispensable for survival in human because there are at least 500 various functions that the healthy liver organ performs.

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Therefore, in the presence of hepatic function failure, every liver cell-based therapy is expected to fulfill these urgent physiological needs. Hence, an extracorporeal liver support system that contains a liver cell unit, i.e., extracorporeal bioartificial liver (EBAL), would be ideal for the substitution of many physiologically relevant functions such as biosynthesis, metabolism, and detoxification.

The EBAL is a cell-based bioartificial liver whose core aim is to provide most of the hepatic functions that are lacking in patients with liver failure. In order that the EBAL carry out these liver-specific functions effectively, it becomes critical that the bioreactor be designed for optimal performance. The role of the bioreactor is to keep these cells working physiologically for a prolonged period of time. In addition to ensure that the bioreactor is compliant with clinical applications, the bioreactor should be reproducible, automatable, controllable, and customizable to cater for individual patient's needs. Further, to scale down this device for the clinical setting, technical challenges such as the *ex vivo* culturing of large masses of cells need to be addressed, so that the bioreactor is suitable both for the cells inside and the treated patient outside. In this review, important considerations, including cell source, mass transfer, the design and construction of the bioreactor, the EBAL configuration, and its relevant clinical evaluation, will be discussed.

II. CELL SOURCE

Liver cells are the biofunction unit in EBAL, which plays the key role in providing organ specific function for hepatic failure patients. Based on knowledge garnered from previous studies involving liver resections, it is known that the maintenance of normal liver function can basically be satisfied with about 20% of the whole liver mass, which has approximately 200 g, that is, 2×10^{10} hepatocytes. Rinkes *et al.*[6] suggested that 10% of the entire amount of liver, about 1.5×10^{10} hepatocytes, is enough to perform the function of the whole mass. At present, most of the EBALs aimed at clinical application are loaded with $(1-2)\times10^{10}$ hepatocytes,[5] which theoretically are sufficient to meet the needs of clinical treatment of liver function failure. However, results from the large phase III clinical trials show that, with the treatment of EBAL, although various biochemical parameters and clinical symptoms can be significantly improved in the patients, the overall survival has not been prolonged significantly. The explanation for such results can be various and complicated. To effectively evaluate the biological function of the EBAL system, the amount of liver cells obviously cannot be the only item to consider. It is necessary to include other critical factors that have an important impact on the outcome of treatment, such as the function level and viability of liver cells before and during the treatment.[7]

A. Hepatocytes' polarity

Hepatocytes, as the other epithelial cells, are highly polarized and are defined as the symmetry of cell membrane with the localization of different membrane proteins and lipids on different membrane domains. Its unique position in the liver served as a barrier between the blood and the bile, and its physiological functions of substances trafficking between sinusoids and bile canaliculi predominantly rely on cell polarity. The generation of hepatocyte polarity involves (i) the establishment of cell-cell and cell-matrix interactions, (ii) the organization of cytoskeleton, and (iii) the sorting and localization of membrane proteins. Both cell-cell and cell-matrix interactions trigger the binding of actin filaments to the adhesive machinery. The associations between adhesive complex and actin filaments (i) reinforce the structure of cell-cell and cell-matrix interactions and (ii) serve as a scaffold to recruit downstream signaling molecules so that the local cue of polarity formation can be propagated to the rest part of the cell.

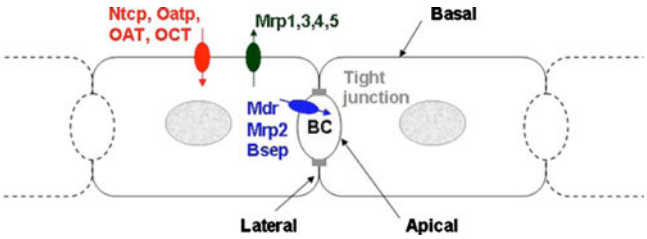

FIG. 1. (Color online) Segregation of membrane domains and transporter distribution in different domains of the hepatocytes.

The cell membrane of hepatocytes can be divided into three distinct domains (Fig. 1): (i) the basal domain, which is faced to the sinusoids for mass exchange with the blood and can also be termed as sinusoidal domain; (ii) the lateral domain, which is the region of direct contact with the neighboring hepatocytes to form cell-cell adhesion; and (iii) the apical domain, which is faced to the bile canaliculi for bile excretion and can be also termed as canalicular domain. The basal and lateral domains are structurally continuous and are commonly termed as basolateral domain. Tight junction served as a structural barrier to separate basolateral and apical domains. Each domain is composed of a distinct pool of functional proteins, including adhesion proteins, receptors, and transporters. Sinusoidal uptake and biliary excretion highly depend on transporters to exert liver functions. In the basolateral domain, Na+-taurocholate cotransporting polypeptides (Ntcps), organic anion transporting polypeptides (Oatps), some family members of multidrug resistance-associate proteins (Mrps), organic anion transporters (OATs), and organic cation transporters (OCTs) are expressed. Ntcp is a sodium dependent transporter for uptake of most conjugated bile salts and certain sulphated steroids. Oatp represents a family of sodium-independent transporters, which is responsible for transport of conjugated and unconjugated bile acids, neutral steroids, some organic anions such as bromosulphophthalein (BSP), limited organic cations, and numerous drugs. OAT and OCT also belong to sodium-independent uptake system. Their substrates remain to be found. Mrps mediate efflux and are adenosine-derived nucleotide (ATP) dependent. Mrp1 is for excretion of drug-glutathione, -glucuronide, and -sulfate conjugates; Mrp3 is for efflux of bile salts; Mrp4 and Mrp5 are for efflux of nucleoside

analog drugs. Section II A 1 will focus on how to enhance the biofunction of EBAL with respect to cell source, cell type selection, cryopreservation, and transport of liver cells.

1. Cell type selection

a. Primary human hepatocytes Primary human hepatocytes are the ideal cells for EBAL. The benefits of using them are obvious; they include better biosafety, the satisfactory level of hepatic biofunction, particularly the ability of providing homologous biologically active substances. Poyck *et al.*[8] compared the metabolic functions of primary human hepatocytes, fetal human hepatocytes, and porcine hepatocytes in their EBAL. They found that the ability of primary human hepatocytes for ammonia removal and urea synthesis was, respectively, two and three times higher than those of porcine hepatocytes. They also discovered that the function level of fetal human hepatocytes for lidocaine metabolism was 3.5 times higher than that of the porcine hepatocytes, and 6.6 times higher than the primary human hepatocytes. However, the fetal human hepatocytes produce an extra amount of ammonia into the culture medium, which is not preferable for EBAL application, but more suitable for the study of drug metabolism.

However, the primary human hepatocytes are scarce, do not proliferate *in vitro*, and their normal functions cannot be maintained optimally *in vitro* for longer than 1 or 2 weeks. Most of the primary human hepatocytes used in the EBAL for clinical trial were collected from discarded tissues proceeding from liver resection. Baccarani *et al.*[9] collected 54 liver tissue blocks from liver resection or from whole liver organs not suitable for transplantation and built up a primary human hepatocyte bank. However, because most of these samples were collected from different diseased tissues, such as cirrhosis, fibrosis, and cholangitis, the isolation was comparatively difficult and the cell yield and viability were not satisfying. In addition, there was a great variation of functional performance among different cell batches. Besides these, the potential contamination with viruses, bacteria, and tumor cells was a major concern in these cells that were destined for EBAL. Hughes *et al.*[10] harvested livers from 20 cardiac arrest donors. The hepatocytes were isolated by the method of collagenase perfusion with the average yield of 2.2×10^6 hepatocytes per gram of liver mass, and the cell viability being up to 52%, which were expected as the cell source suitable for clinical application. But it was showed that both the harvesting and survival rate were significantly affected by the length of time they were exposed to the damage caused by cold ischemia and warm ischemia, meaning that the cell functions still need to be validated in detail before using. Therefore, making a proper choice for the tissue source, improving the technology for isolation and storage, and thereby building up a ready-to-use primary human hepatocyte bank are pertinent for the EBAL.

b. Primary porcine hepatocytes Primary porcine hepatocytes are the preferred and the most widely used animal-derived liver cells for EBAL due to its convenient availability, low cost, and well-established isolation methods. They show up more frequently in clinical reports, even entering into clinical trials of phase II/III. Recently there has been a considerable progress in using the cell source of primary porcine hepatocytes, but there are still a number of drawbacks.

(1) Primary porcine hepatocytes present xenogenic antigens to human body; thus the first issue to be considered when using them in clinical is the immune response generated during treatment. A study by Baquerizo *et al.*[11] found that the levels of antipig IgG and IgM antibodies increased significantly in the serum of the patients who received two or more EBAL support treatments. Schulte arn Esch *et al.*[12] detected the C3, C4, factor B, membrane attack complex (MAC), and γGST in the serum samples collected from the patients treated with primary porcine hepatocytes charged EBAL by ELISA (Sigma-Aldrich). They found that the concentration of C3, C4, factor B, and MAC increased gradually in the course of the treatment. With immunohistological assay, the study also found the human antibodies and the agglutination of the above factors on the cell membrane of porcine hepatocytes, and their concentration intensity can also be enhanced with the increasing number of patient treatment. In addition, the DNA fragmentation of porcine cells increased along with the number of EBAL treatment, which showed that the immune system (especially the complement system of it) in the patients treated with EBAL was triggered to attack the porcine cells loaded inside the EBAL. van de Kerkhove *et al.*[13] reported that primary porcine hepatocytes expressed Gal-alpha (1-3) Gal, which was confirmed to play a critical role in the hyperacute rejection induction in liver transplantation, and could elicit similar reaction in the patients treated with the EBAL containing the xenogeneic cells. To address these problems, some other researchers[14] tried to supply the serum from liver failure patients with a kallikrein inhibitor, nafamostat mesilate, to prevent the immune reaction against porcine cells, which significantly reduced the injury of porcine hepatocytes. In addition, the production of Gal-alpha (1-3) Gal gene knockout pigs will be a promising solution for the xenogeneic immune rejection.[15–17]

(2) Pig endogenous retrovirus (PERV) is a class of C-type retroviruses common in pig tissue and cells. Although there is no case report of PERV infection caused by the direct exposure of human plasma to porcine cells in the patients who received the treatment of EBAL loaded with porcine cells, the potential dangers that it poses are still a major concern.[18] Present studies show that PERV can infect primary human liver cells cultured *in vitro*,[17] the endothelial cells, embryonic kidney cells (293 cells), and the other various cell lines from rhesus monkeys, orangutans, baboons, and other primate animals can also be infected.[19] However, when treated with high doses of PERV and a strong immune inhibitor at the same time, none of animals, including Romania monkey, pigtail monkey, baboons, and other primates, was detected to be PERV infected.[20]A study transplanted porcine-derived islet cells into immunodeficient mice.[21] The mice were

divided into two groups, which were, respectively, injected with normal human serum or phosphate buffer solution (PBS) 2 days before the transplantation. Graft infiltrating lymphocytes (GILs) from both groups were harvested and analyzed at day 21 after transplantation. The results showed that the PERV was positive in three of the five mice in the group with PBS pretreatment, but was not detected in the mice of the human plasma pretreatment group. It was speculated that PERV infection can be blocked by the normal human plasma due to the neutralization effect from the natural antibodies against the heterologous antigen inside the plasma. Despite the fact that the *in vivo* results can be attributed to the protective effect of the body's immunological system, it does not exclude the fact that there might be a high risk of PERV infection in the patients treated with porcine cell charged EBAL.

To minimize the risk of PERV transmission during therapy, the semipermeable polymer membranes are normally used as barriers in the EBAL devices.[22] Generation of non-PERV pig by the technology of genetic interference can be a promising solution as well;[23] as much as 90% reduction in PERV RNA and protein level was reported.[24] However, due to the broad existence of PERV, complete elimination of PERV in pigs can be too difficult to achieve. Therefore, other solutions,[25,26] such as administration of antiretroviral protein reverse transcriptase/protease inhibitors (e.g., zidovudine),[27–29] and development of PERV vaccine and anti-PERV antibodies[30] are adopted to meet the needs.

c. Human liver cell lines Among the myriad strains of human liver cell lines, C3A cell line is currently the only one that has been permitted to be used in the clinical trial of EBAL. C3A is derived from a clone of HepG2 cell line. It has a higher level of albumin secretion and CYP450 activity than HepG2. However, compared to primary porcine hepatocytes, C3A has a lower metabolism ability, particularly to ammonia.[31] Mavri-Damelin *et al.*[32] used the labeled $N_{15}H_4CL$ to detect the ammonia removal capacity of C3A and found that the urea generated did not contain the labeled N_{15}. By further analysis of reverse transcription polymerase chain reaction (RT-PCT) and western blotting, the enzymes, ornithine carbamoyl-transferase, and arginase for urea cycle were lacking in C3A, which shows that C3A cells cannot perform the normal ammonia metabolism, instead they produce ammonia through the pathway outside the urea cycle. For liver failure patients who need EBAL support, the metabolic transformation capacity is more compulsory and beneficial than biological synthesis. This is particularly critical for the ones with hepatic encephalopathy, to whom one of the upmost therapeutic strategies is the efficient removal of the toxicant ammonia from systemic circulation. This could be one of the important reasons why some EBAL systems using C3A as their biofunction performer were not able to obtain significant efficacy in clinical treatment.

B. Coculture

In a normal liver tissue, the cell-cell, cell-extra-cellular matrix (ECM), and cell-microenvironment interactions have been widely explored and acknowledged for their major role in the maintenance of hepatic functions. Many studies tried to simulate the physiological organization of liver tissue by recruitment of the nonparenchymal cells (NPCs) into the liver function unit in EBAL, and it has been shown that the hepatic functions can be preserved for long term in the coculture system.[33–37]

Some soluble factors, which are believed to have important influence on the differentiated phenotype of hepatocytes, can continuously be generated as long as the heterogeneous cells' interactions are maintained in the coculture system. Chia *et al.*,[38] cocultured a rat primary hepatocyte with a mouse embryonic fibroblast cell line, NIH3T3 in a three-dimensional (3D) microencapsule formation and found that the direct interactions between the cells and their secretion of various soluble factors play an important role in enhancing the hepatocytes' function. Among them, transforming growth factor (TGF)-β1 is prominently necessary for the effects. Ohno *et al.*[37,39] found that in the hepatocyte-endothelial cell-sheets layered coculture system, drug metabolism related genes expression in the hepatocytes is upregulated, and the ECM molecular composition and cell-adhesion molecules changes in the system correlate with the genes expression in a time-course pattern. The study suggested that the coculture system can generate the beneficial ECM to maintain the hepatic function *in vitro*. Another study by Khetani *et al.*[40] explored the correlation between the cocultured liver function responses and the genes expression profile in different supporting cells. Through further validation, two candidate genes were found to play an important role in the modulation of liver function in the coculture system, decorin related to upregulation, while N-cadherin for the downregulation. But neither of them alone can produce the maximal effects in the system, which indicated that there exists the multifactorial regulation in the cell-cell interaction. Recently, they further examined the modulation effect of T-cadherin on liver cells' function *in vitro* and found that overexpression of T-cadherin in the cocultured cells can significantly enhance hepatocytes' function in a dose-dependent manner.[41] All the above studies elaborated that in the coculture system, NPCs help hepatocytes maintain a specific phenotype by expression of important bioactive molecules.

C. Hepatocyte cryopreservation for EBAL

To meet the requirements of clinical practice, the supply of cell source for EBAL must be rapid, sufficient, and ready to use. Preservation of large quantities of hepatocyte in advance is necessary to ensure that sufficient cell source is readily available for clinical purposes. Cryopreservation at −80 or −196 °C remains the most effective method for long-term preservation of liver cells. However, cell viability and function of primary hepatocytes can be significantly damaged after the frozen/recovery procession. Optimization of the cryopreservation/thawing procedures to improve the cell

survival and the recovery of liver function is critical to address the requirement of cell source in EBAL bioreactor construction. The strategies currently taken include (1) refinement of the isolation technology, and incubation of the isolated cells with medium containing antioxidants, at low temperature, before freezing.[42–44] This can effectively reduce the isolation damage to the cells and improve their quality prefreezing. (2) Modification of the formula of the cryopreserving medium and the protective agent. Currently the University of Wisconsin (UW) solution and dimethyl sulfoxide (DMSO) are still recognized as the best frozen liquid and the most important permeable cryoprotectant, respectively.[45] Making amendment to the cryopreservation medium by reducing the concentration of bovine serum, adding one or a combination of membrane stabilizers (such as trehalose and taurine),[46,47] biological antioxidants (such as catalase, N acetyl cysteine, glutathione, and fetal bovine serum),[48,49] apoptosis inhibitor (such as Z-DEVD-FMK and IDN-1965)[50,51] with appropriate concentration, can significantly improve the freezing effect. (3) Optimization of the store configuration of cryopreservation. Hepatocytes are anchorage-dependent cells; the maintenance of their functions and activities depends on the support from ECM and cell-cell communication.[52] Cryopreservation configurations such as monolayer collagen, sandwich collagen, microcarrier, and microcapsule stock of hepatocytes showed better post-thawing viability and function than the suspension cell cryopreservation. For example, HepatAssist system employs the microcarrier adhesion of hepatocytes for cryopreservation. (4) The other factors in the process of cryopreserving/thawing, such as type of vials,[53] cell density,[54] cooling rate, and recovery procedures, also involve in the preservation effect.[45]

Ultralow temperature cryopreservation, which normally keeps the storage in liquid nitrogen, is a relatively mature technology for long-term cryopreservation of liver cells; but there are still shortcomings such as obvious damage of cell viability and function, complicated operation procedure, high cost of the programmable freezing, and inconvenience transportation. Therefore, to avoid injuries caused by cryopreservation such as extra- and intracellular ice formations, stresses rising from solution effect, and dehydration, some researchers focus on exploring the room/low temperature preservation technology, to satisfy the requirement of simplicity, being ready to use, transportation convenience, and effective maintenance of cell function.

Subzero nonfreezing temperature (SNFT) is the temperature range between 0 °C and freezing point. Ultracold temperature is the range between the temperatures below freezing and the temperature at which the solution begins to form ice crystals. For UW solution, the SNFT is 0 °C–(−4) °C, and the ultracold temperature is −1 °C–(−4) °C. SNFT and ultracold temperatures not only reduce the maximum metabolic activity of cells, but also avoid freezing damage caused by ice crystal formation. Theoretically it is an ideal way for cryopreservation of liver cells. Yoshida *et al.*[55] preserved isolated rat liver with UW solution. The treatment groups were divided into 4 (control) and −0.8 °C groups. The results showed that in the −0.8 °C group, the content of ATP and total adenine nucleotide (TAN) was significantly higher, and the lactate dehydrogenase (LDH) release was lower than that of the control group. Rodriguez *et al.*[56] preserved rat liver cells with UW solution, which were divided into 0, −4, and −4 °C plus nonpermeable cryoprotectant butanediol. After cold storage for 120 h, the survival rate and the ATP content of cells at −4 °C were significantly increased, higher than those of 0 °C, while LDH release and lactic acid products were decreased significantly. In addition, supplementing butanediol to UW solution can further improve cell viability and intracellular ATP content at −4 °C.

On the other hand, further progress was made in the preservation of hepatocytes at room temperature. Epigallocatechin-3-gallate (EGCG) as the major component in green tea polyphenol is found to have wide bioactivities, such as antioxidant, anti-thrombotic, etc. Miskon *et al.*[57] explored using EGCG as a key compound in the room temperature preservation of hepatocytes. 2×10^9 porcine hepatocytes were seeded in the polyaminourethan coated polytetrafluoroethylene (PTFE) scaffold in a radial-flow bioreactor. After perfusion culture for 4 days, the supply of culture medium, oxygen, and carbon dioxide was removed, and 0.25 mg/ml EGCG was added into the medium. The preserved hepatocytes are kept at room temperature for another 4 days, then washed with the incubation medium and went on culturing for 6 days. It was found that after the room temperature preservation, the hepatocytes still maintained good morphology, and its functions of ammonia metabolism and albumin synthesis, respectively, recovered to the $72\pm16\%$ and $98\pm32\%$ of the levels before treatment.

Vitrification refers to the addition of a variety of high concentrations of cryoprotectants (which act as antifreeze) to cells to produce the right degree of dehydration, and then cool rapidly (often more than 1500 °C/min). With high concentration of antifreeze and liquid viscosity increases, there can be a formation of an extremely thick glasslike substance in the amorphous state to effectively prevent the formation of intracellular ice crystals during the rapid cooling and thawing process. The operation protocol is simple, fast, and without the need of an expensive cooling device.[58] Therefore, vitrification has been widely used in stem cell,[59–61] pancreatic islet cells, kidney, bone marrow stromal cells,[62] liver cells,[63–65] and other tissue cryopreservation.

Although many studies show that vitrification is more effective than conventional slow freezing method, the toxicity due to high concentrations of permeable cryoprotectant and the osmotic injury produced during removal of refrigerant are the two major shortcomings of the method. Further optimization of the cryopreservation procedures for better protection of the cells and the formulation of the frozen liquid are the core issues in this field. Adding various substances into the cryopreservation solution, such as sugars, hyaluronic acid, Relaxin, salt, and antifreeze proteins, can improve the cooling effect.

III. MASS TRANSFER IN EBAL BIOREACTOR

A. Oxygen supply

1. Optimal oxygen tension

The most favorable oxygen tension for hepatocytes' culture for EBAL usage can be defined as a range of intracellular oxygen tension that enables hepatocytes to closely resemble their *in vivo* functionality. Ultimately oxygen is consumed by the hepatocytes to produce ATP in the mitochondria or in other oxidative process such as enzymatic addition of oxygen by Cytochrome P450. In the liver itself, the oxygen tension varies in different regions and plays a significant role in compartmentalizing the metabolic and detoxification functions of hepatocytes, known as liver zonation.[66–68] A concise review of the role of oxygen in liver zonation can be found in a paper by Jungermann and Kietzmann.[68]

The oxygen tension of the blood in the sinusoids are 60–70 mm Hg in the periportal region, 35–60 mm Hg in the pericentral region, and 25–35 mm Hg in the perivenous region.[69] However, due to the presence of sinusoids and the space of Disse in between the blood and the hepatocytes, the pericellular (cell-surface) oxygen tension of the hepatocytes is approximated to be 5 mm Hg lower,[70] while the intracellular oxygen tension is suggested to be 15 mm Hg lower.[68,71] Hence the physiological pericellular and intracellular oxygen tensions of hepatocytes are about 20–65 and 10–55 mm Hg, respectively.

At a hyperoxic oxygen tension, hepatocytes might undergo oxidative stress and its functionality and viability compromised. Mitochondria and cytochrome P450 are the well-known producers of active oxygen species such as superoxide and hydrogen peroxide.[72,73] These active oxygen species can react with almost any cellular component and possibly disrupt their structure or functioning.[74] In a greater than normal oxygen tension, the inherent protective mechanism of the cells (the antioxidant enzymes) could be overwhelmed resulting in cytotoxicity.[75]

Many researchers find the reported optimal range *in vitro* oxygen tension of hepatocytes culture from these following studies as controversial.[76,77] Yanagi *et al.*[76] found that a high oxygen concentration of 460 μM (387 mm Hg) of oxygen improves the metabolic activities of the hepatocytes in a packed bed reactor significantly, and Custer and Mullon[78] reported that no oxygen toxicity is observed in the range 152–532 mm Hg. On the other hand, Nishikawa *et al.*[79] found that hepatocytes cultured under 20% atmospheric oxygen tension (152 mm Hg) perform most effectively, while Miyazaki *et al.*[80] found that hepatocytes cultured under normal air (160 mm Hg) experienced hyperoxic condition that compromises their viability. Likewise Badera *et al.*[81] showed that rat hepatocytes undergo oxidative stress when cultured at atmospheric oxygen tension of 160 mm Hg, and Suleiman and Stevens[75] found that above 132 μM or 111 mm Hg (using oxygen solubility value of 1.19 nmol cm^3 mm Hg^{-1})[82] oxygen concentration, the viability of the hepatocytes gradually decreases in the early stage of culture probably due to oxidative damage.

To overcome this ambiguity and seemingly incongruous data, a possible solution is to report instead the pericellular oxygen tension. A study by Wolff *et al.*[83] indeed found that incubation gas oxygen tension is insufficient to predict the Epo production rate of human hepatoma cell lines, instead it found a clear correlation between Epo production and pericellular oxygen tension. Suleiman and Stevens' value should be somewhat closer to the pericellular oxygen tension as they use a well-stirred culture medium, though an experiment in 1970 has shown that oxygen depletion region around the cells still occurs despite vigorous mixing.[84] It could also be the case for the value of Badera *et al.*, as they used a collagen-coated oxygen-permeable bottom directly below the cultured cells, depending on the thickness of the collagen layer. Hence, the upper threshold for pericellular oxygen tension where no oxygen toxicity occurs is probably in the 90–111 mm Hg range.

Studies by Subramanian *et al.*[71] and Schumacker *et al.*[85] present many important insights about hepatocyte metabolic activity, in relation to the oxygen tension of the culture media. Note that the following reported oxygen tension values were measured from well-stirred culture media. Essentially they found that the cellular [ATP] decreases when the oxygen tension is slowly decreased below 100 mm Hg. As the availability of the oxygen decreases, mitochondria uptake rate of oxygen decreases and ATP production decreases. To avoid eventual total depletion of the cellular ATP, as would be the case when consumption rate is larger than the production rate, the intrinsic ATP demand of the cell is downregulated. To maintain viability, only the "nonessential" ATPases are (reversibly) suppressed while ATPases that are crucial for cell viability (e.g., Na–K ATPases) are maintained.[71] This selective suppression can be possibly explained by the varying ATP affinities of different enzyme systems.[85] It can also be seen from their results that the intracellular [ATP], and not the oxygen tension, correlates closely with the oxygen uptake rate of the cell. It is unknown whether the level of metabolic activities (and the oxygen uptake rate) of the hepatocyte is influenced directly by the intracellular [ATP] and/or through an (unknown) oxygen sensor that is able to modulate specific genes expression.

It is possible that the above-mentioned modulation of intracellular [ATP] through oxygen availability is involved in the creation of liver zonation *in vivo*. The suppression of acetaminophen detoxification at 20 mm Hg oxygen tension[71] is consistent with the finding of a study using intact liver that the *in vivo* rates of glucuronidation and sulfation are greater in the periportal region.[86] Other periportal-zonated functions, gluconeogenesis, urea, and albumin synthesis,[68] are compromised at lower oxygen concentration.[87,88] On the other hand, certain metabolic functions such as erythroprotein (EPO) production,[89] glycolysis,[68] CYP1A1,[90] and CYP2B expression[66] are improved under lower (perivenous) oxygen tension.

In conclusion, it is desirable that the pericellular oxygen tension of the hepatocytes in a perfused bioreactor mimics the *in vivo* physiological counterpart so as to be able to perform liver functions found in various zones. Exceeding it could cause oxidative stress, while having less than it will cause undesirable extensive suppression of functions and even cell death.

2. Oxygen uptake rate of hepatocyte

To be able to supply an appropriate amount of oxygen for hepatocytes' culture, the knowledge of hepatocytes' oxygen uptake rate (OUR) is important. The OUR of hepatocytes is often modeled according to Michaelis–Menten (MM) kinetics as it has been shown to reasonably match the experimental data.[91] This model represents the manner in which the OUR of the hepatocyte decreases in response to a decrease in local oxygen tension, as have been discussed earlier. The values of V_m, the maximum OUR, and K_m, the oxygen tension that corresponds to half V_m OUR, can be found through experiments.

The OURs of hepatocytes have been found to vary between species, time phase of culture, cell density, and usage of NPCs. Balis *et al.*[92] measured the V_m of porcine hepatocytes to be 0.9 nmol s^{-1} million cells^{-1} at day 4, which decreased gradually to 0.31 nmol s^{-1} million cells^{-1} by day 15, while the K_m value is 2 mm Hg at day 2, increased to 8 mm Hg at day 8, and decreased to 4 mm Hg by day 15. On the other hand, Rotem *et al.*[93] experiment revealed that the V_m of rat hepatocyte was about 0.34 nmol s^{-1} million cells^{-1} during the first day and stabilized to 0.27 nmol s^{-1} million cells^{-1} afterwards.

The higher V_m at the early phase of culture is attributed to the high energy requirement for the hepatocytes to spread.[93,92] The application of this knowledge has been shown by a study in which immobilization efficiency of hepatocytes improves at higher oxygen concentration (30% or 40% oxygen in the atmosphere).[94] This seems to contradict Suleiman and Stevens' finding that cell viability decreases significantly at and above 20% atmosphere oxygen during the early culture period due to oxidative damage.[75] A possible explanation is that Suleiman and Stevens pre-equilibrate their culture medium with the atmosphere, creating immediate hyperoxic pericellular oxygen tension, while Yanagi and Ohshima did not, and hyperoxic pericellular oxygen tension was never developed due to oxygen gradient formation in the culture medium.

The effect of cell density toward the OUR of hepatocytes is still not clear. Guarino *et al.*[95] found that hepatocytes are metabolically more active and hence consume more oxygen when the cell to cell contact is enhanced by increasing cell culture density. On the other hand, Patzer[77] theorized that a high cell density closely resembles the natural *in vivo* condition and creates less stressful condition for the hepatocytes, hence explaining the observed decrease of the OUR in some experiments when the cell seeding density is high. Cho *et al.*[96] showed that the V_m of hepatocytes cocultured with 3T3-J2 fibroblasts varied according to the hepatocyte to fibroblast ratio, with lower ratio causing an increase in hepatocyte V_m. Interestingly, the resultant increase in the OUR was observed to correlate well with the increase in urea synthesis and albumin secretion. It remains to be seen whether this increase in V_m correlates with intracellular [ATP], as has been discussed previously.

B. Effect of shear stress on hepatocyte culture

In vivo, blood flow will directly induce shear stress on hepatic sinusoids, and through the fenestra, on hepatocytes and the NPCs. These cells are known to response to shear stress probably in independent and interdependent manners. A well-known example is the induction of PAI-1 gene expression in hepatocytes[97] and nitric oxide (NO) secretion by endothelia cells[98] in response to increased shear stress following a partial hepatectomy, in which both are involved in liver regeneration process.

The value of shear stress on the sinusoids can be as high as 20–40 dyn/cm^2.[99] While the values for the rest are unknown,[97] we can expect them to be significantly lower, considering the shielding effect of sinusoids and the presence of space of Disse. In a study using liver slices, moderate shear stress after 12 h was found to maintain cytoplasmic membrane integrity and improved albumin production unlike no shear stress or high shear stress condition.[100] The exact value of moderate shear stress is unclear but it was stated as between 0.5 and 20 dyn/cm^2.

The creation of an EBAL necessitates the use of perfusion culture to improve mass exchange (e.g., oxygen supply or waste removal), which unavoidably imposes shear stress on the cells. Though many studies have been done to find out the acceptable or beneficial shear stress values for hepatocytes' culture, no consensus has been achieved yet. Indeed' as we discuss further into this topic' we might find that the effect of shear stress for different *in vitro* culture setups (e.g., cell species, presence of NPCs, or fluid-cell membrane barrier), culture period, and functions [e.g., cytochrome P450 superfamily (CYP) activity or urea synthesis] might be inherently different.

In a study by Kan *et al.*[99] using rat hepatocytes' coculture system, shear stress (4.7 dyn/cm^2) was found to induce NPCs' secretion of extracellular matrix, which in turn enables hepatocyte-NPC reorganization into aggregates that perform significantly better functionally. This enhancement is attributed to a better cell-cell interaction and the NPC-secreted extracellular matrix. Interestingly, by having 7 day preperfusion stationary culture period, the hepatocytes perform better as compared to having lesser period or none. This finding has to be applied carefully for other culture conditions, where NPCs are absent, or sinusoids, which in itself also influence hepatocytes functioning in response to shear stress,[99] are present, or overlaying membrane, which might restrict aggregate formation, is used.

Short-term (3 days) study of rat hepatocytes' coculture finds that lower wall shear stress group (0.01–0.33 dyn/cm^2) performs better than higher shear stress group (5–21 dyn/cm^2) in terms of urea and albumin

production.[101] Looking at the albumin production for 10 day perfused culture under 0.33 dyn/cm^2, a sudden significant improvement was not observed on any day. This is unlike the result of Kan *et al.*, which shows a significant improvement of albumin production on day 4. This could mean that 0.33 dyn/cm^2 is too low to induce NPCs' secretion of extracellular matrix, which allows beneficial aggregate formation. Further study is needed to compare the long-term (7 days or more) hepatocytes' coculture functioning under low and high shear stresses. As for detoxification function, a study of rat hepatocytes' coculture found that a shear stress as high as 10 dyn/cm^2 does not adversely affect CYP1A1 activity for at least 12 h.

A recent study using human hepatocytes (homotypic) cultured in perfused microchip bioreactors under various shear stresses of 1.4–60 dyn/cm^2 has found that albumin production is optimized at the lowest shear stress for at least 3 days.[102] The study has also found that the cell shape of hepatocytes remained in the same shape as in static culture when cultured below 5 dyn/cm^2.

Overlaying membrane is utilized in a sandwich system as a polarity cue to improve hepatocyte functioning. It is possible that the membrane acts as a mechanically shield and reduces the shear stress acting on the hepatocytes.[103] This could work in two ways by partially "absorbing" the force generated by the shear flow or by smoothening the fluid-solid interface. To elaborate the latter point, first we have to understand that on the cell surface itself, there exists maximal shear stress region (usually around the cell apex) due to uneven cell contours.[104] The presence of membrane can arguably help to even out the contour and change the shear stress distribution pattern.

Interestingly, our experiences with sandwich culture indicate that sandwich culture could be more sensitive to shear stress. This can probably be explained if hepatocyte employs focal adhesion (FA) as part of mechanosensory device. Since shear stressed membrane is directly attached to ECM, higher tension might be generated to the focal adhesion than normally (absence of overlaying membrane) would with the same fluid flow rate. FA has been implicated as the mechanosensors in many cells.[105,106]

C. Computer fluid dynamic (CFD) modeling of EBAL

CFD is a computational technology that enables the creation of fluid model on which the desired physical and chemical phenomena can be imposed through the application of appropriate mathematical equations. CFD involves simultaneously solving fluid motion equation (the Navier–Stokes equations are commonly used) with other applicable equations (e.g., mass transfer equation) using the appropriate boundary conditions. A fundamental aspect of CFD is the use of computer to solve the representative mathematical equations with numerical methods that involve discretization process (using finite difference, finite element, and finite volume methods). CFD inherently possesses a weakness that only approximate solution can be achieved, though with proper discretization reasonably accurate solutions can be obtained. Theoretically CFD enables the simulation of fluid with any geometry under any physical and chemical phenomena.

Typically, CFD simulation for EBAL is used to find flow velocity, shear stress, and oxygen tension profile, which sometimes are impossible to obtain experimentally. Moreover, CFD simulation enables optimization of EBAL design without the need of physical testing, which translates into a faster and cost-effective product development. Validation of model commonly involves comparing the outlet oxygen tension or the oxygen consumption rate between the simulated and experimental results.

With commercially available CFD software, such as ANSYS CFD (ANSYS Inc., Canonsburg, PA), COMSOL MULTIPHYSICS (COMSOL Inc., Burlington, MA), or ADINA (ADINA R&D Inc., Watertown, MA), bioreactor models with complex features and geometry have been simulated, as will be listed out in the subsequent paragraphs. Though usually significantly more costly than MATLAB, it has the advantages of having a user-friendly interface, an in-built graphical interface, and in-built mathematical equations and solvers.

3D CFD simulation of a flat-plate bioreactor using FLUENT (ANSYS Inc., Canonsburg, PA) reveals the possible limitation of two-dimensional (2D) modeling.[107] In the real case (3D), the fluid velocity varies in the width (z) direction as well, and hence the ratio of channel height to width has to be significantly low for the 2D assumption to be reasonable. Specifically, for oxygen transport problem, the 2D model solution will be significantly different if the Damkohler number is below 0.5 and the Peclet number is below 1000.

2D CFD simulation of hollow fiber bioreactor using FEMLAB (now known as COMSOL MULTIPHYSICS) was done with axisymmetry but without the radial-only diffusion assumption.[108] Besides implementing MM oxygen consumption of hepatocytes, hemoglobin model as an oxygen carrier is also included.

Rotating hollow fiber bioreactor for artificial liver has been simulated using FLUENT, in which 2D model was created to find the optimum rotating speed for best multiphase (microcarrier-attached aggregated cells and culture medium) mixing, and 3D model was created to simulate the oxygen tension profile and fluid-induced stress of the floating aggregates.[109]

Radial-flow bioreactor consisting of a stack of circular microgrooved glass substrates was simulated with ANSYS CFD FLOTRAN.[110] This bioreactor has a relatively complex flow pattern in which medium flows from the peripheral edge toward the central vertical channel where the axisymmetry line is located at. The role of microgrooves to reduce shear stress imposed on hepatocytes/fibroblast cocultures has been shown through the use of simulation. In addition, the oxygen tension profile has also been simulated and validated by comparing the simulated and experimentally measured outlet oxygen tension. In this simulation, the OUR of the cells is assumed to be a constant equal to the V_m value, which is reasonable if the oxygen tension is significantly larger than the K_m value.

A macrofabricated array bioreactor for 3D liver culture

was simulated using ADINA.[110] The hepatocytes are seeded inside microchannels in which culture medium is perfused through them. 3D models of the whole bioreactor and also a representative tissue-filled microchannel were created to find the relevant shear stress values. The hepatocyte tissue inside the microchannel is modeled as a rigid solid with the no slip boundary condition.

IV. EBAL BIOREACTOR CONFIGURATION

The use of EBAL in treatment of liver failure is based on the assumption that detoxification, intermediate metabolism, and macromolecule synthesis function can be provided by hepatocytes cultured in the bioreactor of the EBAL.[5] However, the long-term maintenance of hepatocyte differentiated functions *in vitro* remains a challenging field. Since hepatocytes are anchorage-dependent cells, various biomaterials for *in vitro* hepatocyte culture such as membrane, microcarriers, polymeric matrix, or scaffold are incorporated in bioreactor design for cell attachment. There are mainly four different bioreactor configurations under current investigation: hollow fiber cartridges, encapsulation system, packed bed, and flat plate. The features of eight EBALs under clinical trials are summarized in Table I.

A. Hollow fiber cartridge

Hollow fiber system is the most common bioreactor configuration. Generally, a hollow fiber bioreactor consists of a cylindrical column with hundreds of thousands of hollow fibers aligned longitudinally through the column. Cells are cultured outside the hollow fiber while culture medium or patient's blood/plasma is perfused in the fiber lumen along the hollow fiber. The hollow fibers are made from semipermeable membranes, which allow mass transfer across the membrane between cells and perfusate. However, many research groups modified the basic design of hollow fiber bioreactor to improve the performance.

The majority of EBALs under various stages of clinical trials are based on hollow fiber configuration. They use different culture techniques for cell anchorage outside the hollow fibers. In an extracorporeal liver assist device (ELAD) developed by Ellis *et al.*,[111] human hepatocyte cell line, C3A, is directly seeded and grown to confluence in the extraluminal space of hollow fibers. The Excorp Medical bioartificial liver support system (BLSS) uses primary porcine hepatocytes as cell source. Cells are mixed with collagen to infuse into hollow fiber bioreactor so that collagen gel matrix is formed in the extracapillary region for cell anchorage.[112] Detry *et al.*[113] developed HepatAssist 2000, in which collagen-coated dextran microcarriers are used for porcine hepatocytes attached and are housed in the extracapillary compartment in the bioreactor. The Academic Medical Center (AMC) EBAL system loads a nonwoven hydrophilic polyester matrix in the extrafiber region in the bioreactor for hepatocytes' attachment while the inner-lumen of hollow fibers are used for oxygen supply.[114] Sauer *et al.*[115] developed a modular extracorporeal liver support (MELS) with a more complex interwoven hollow fiber cartridge. Three different bundles of hollow fibers are interwoven to form a three-dimensional framework so that each bundle can perform separate functions such as plasma inflow, plasma outflow, and oxygen supply. Hepatocytes are cultured with a collagen matrix outside, or between, the hollow fibers. In this design, each hollow fiber supplies nutrients for only several hepatocytes so as to reduce nutrients' gradient in the perfusion. The system also allows hepatocytes' coculture with other NPCs to form a tissuelike structure, and the formation of bile canaliculi was observed. In China, the preclinical or clinical trials of hollow fiber EBALs are conducted by several groups, such as the TECA type hybrid artificial liver support system (TECA-HALSS) developed by Chinese People Liberation Army (PLA) general hospital in Beijing,[116] the hybrid bioartificial liver (HEBAL) by the Nanjing University,[117] the novel bioartificial liver (NEBAL) by the Zhejiang University,[118] and the bioartificial liver support system (BALSS) by the Southern Medical University.[119] The common feature shared by these EBALs is the combination of a hollow fiber-based system with other nonbiological modules such as charcoal absorbent or plasma exchange modules to improve treatment outcome.

Three hollow fiber EBALs with distinct feature are in preclinical or *in vitro* test. The LIVER-X 2000 system developed in the University of Minnesota has hepatocytes seeded in the lumen of a hollow fiber instead of the extraluminal space while patient's blood was perfused between fibers.[120] Jasmund *et al.* in Eberhard Karls University developed the oxygenating hollow fiber bioreactor (OXY-HFB) in which hepatocyte seeding and blood perfusion are both in the extrafiber space, allowing direct contact of hepatocytes and blood.[121] The hollow fibers are used for oxygen supply and temperature control. Mizumoto and Funatsu[122] applied a centrifugal force for hepatocytes' inoculation in their liver lobulelike structure (LLS) module EBAL so that hepatocytes can form an organoid structure in the outer space of hollow fibers.

B. Packed bed system

Packed bed, originally used as a term in chemical engineering, refers to a hollow vessel that is filled with packing materials. In application of EBAL, this configuration can be used to fill matrix for hepatocyte attachment and perfused with medium or patient's blood/plasma. Various packing materials for hepatocyte entrapment, such as microchanneled polyurethane foam,[123] polyvinyl resin cubes,[76] alginate beads,[124] porous hydroxyapatite beads,[125] and polyester fabric cell scaffold[126] were explored. The radial-flow bioreactor (RFB) EBAL system, designed in the University of Ferrara, is the only packed bed configuration under clinical trial.[127] In the bioreactor, hepatocytes are entrapped within the woven polyester microfibers and patient's plasma is perfused from the center to the peripheral of packed bed allowing the direct contact of hepatocytes to plasma, whereas in some other systems, the perfusion pathway is reversed.

TABLE I. EBALs under current clinical trials.

EBAL system	Research group	Bioreactor configuration	Cell source and amount	Cell culture technique	Treatment time	Clinical perfusate (plasma filtration rate, ml/min)	Bioreactor flow rate (ml/min)	Oxygen supply
ELAD[a]	Ellis *et al.*	Hollow fiber	Human C3A line 400 g	Hollow fiber attached aggregates	Up to 168 h	Blood	15–200	Prebioreactor
Hepat-Assist[b]	Detry *et al.*	Hollow fiber	Cryopreserved porcine $(5–7)\times10^9$	Microcarrier attached aggregates	45–89 h	Plasma (50)	400	Prebioreactor
BLSS[c]	Mazariegos *et al.*	Hollow fiber	Freshly isolated porcine 600 g	Collagen gel entrapped	12 h	Blood	100–250	Prebioreactor
AMC[d]	van de Kerkhove *et al.*	Hollow fiber	Freshly isolated porcine 12×10^9	Polymeric matrix attached aggregates	Up to 24 h	Plasma (40–50)	150	By capillary inside bioreactor
MELS[e]	Sauer *et al.*	Hollow fiber	Freshly isolated porcine/human 600 g	Tissuelike organoid	7–74 h	Plasma (31)	100–200	By capillary inside bioreactor
RFB[f]	Morsiani *et al.*	Packed bed	Freshly isolated porcine 200 g	Microfiber entrapped aggregates	Up to 24 h	Plasma (22)	200–300	Prebioreactor
TECA-HALSS[g]	Xue *et al.*	Hollow fiber	Freshly isolated porcine $(10–20)\times10^9$	Cell in outer space of hollow fiber	Up to 5 h	Plasma (not reported)	Not reported	Prebioreactor
HEBAL[h]	Ding *et al.*	Hollow fiber	Freshly isolated porcine 10×10^9	Cell in outer space of hollow fiber	6 h	Plasma (not reported)	Not reported	Not reported

[a]Reference 111.
[b]Reference 113.
[c]Reference 112.
[d]Reference 114.
[e]Reefrence 115.
[f]Reference 127.
[g]Reference 116.
[h]Reference 117.

C. Encapsulation system

In an encapsulation system, hepatocytes are enveloped in a polymeric matrix to form a small capsule and encapsulated hepatocytes are packed in a chamber for perfusion. Various materials have been used for hepatocyte encapsulation, including hydrogels,[128] alginate,[129] and copolymer such as hydroxyethyl methacrylate-methyl methacrylate (HEMA-MMA).[130] In some systems, hepatocytes form spheroids before entrapped into a capsule,[131,132] because there are evidences showing that spheroid enhances cell-cell interaction,[133] and facilitates the formation of bile-duct structure between cells to improve cell functions. However, there are no EBAL of encapsulation configuration under clinical trial so far.

D. Flat-plate system

Hepatocytes' monolayer culture on the substratum such as collagen, laminin, fibronectin, or Matrigel is a common *in vitro* culture method. Overlay of a matrix gel layer on the top of monolayer culture to form a sandwich configuration stabilizes hepatocytes' cuboidal structure. The establishment of hepatocyte polarity and maintenance of differentiated functions for several weeks can be achieved.[103,134] The pioneer to employ flat plate configuration in EBAL application is Uchino *et al.*[135] in 1987 who stacked 200 hepatocyte monolayers on a collagen-coated glass plate and confirmed the efficacy of EBAL treatment in anhepatic dogs to extend their survival time. They further improved the results by replacing monolayer culture with sandwich culture, and better hepatocyte functions in stacked collagen sandwich bioreactor module were evident.[136] A full-scale flat membrane bioreactor (FMB) based on sandwich culture was developed in Germany.[137] Each stackable FMB module is 1150 cm^2 and up to 50 modules can be perfused in parallel. Park *et al.*[138] developed a radial-flow bioreactor with stacked microfabricated grooved substratum in which the grooved glass plate protected hepatocytes from negative impact of flow shear stress. Sandwich culture is considered to be one of the best *in vitro* hepatocyte culture models. However, the application of sandwich culture in EBAL bioreactor design is stagnant since several technical difficulties, such as relatively low cell housing capacity compared to other configurations and labor-intensive bioreactor assembly, need to be addressed in future bioreactor design.

E. Microfluidic chip based system

Microfabrication based microfluidic system for hepatocyte culture has emerged as a promising area for various hepatocyte *in vitro* applications including EBAL. The fine control of hepatocyte microenvironment, which is essential to maintain hepatocyte differentiated functions, is made possible at microscales in microfluidic system. The small fluid volume in microfluidic perfusion allows more efficient mass transfer in terms of delivery and removal of soluble substance.[139] Lee *et al.*[140] created an artificial liver sinusoid with a microfluidic endothelial-like barrier and rat or human hepatocyte can be cultured within the barrier. Hepatocyte viability and drug metabolism functions can be maintained for 7 days in the system. The first attempt to design a microfluidic system based bioreactor for EBAL was by Leclerc *et al.*[141] from the University of Tokyo. Ten microfabricated polydimethylsiloxane (PDMS) chips were stacked to constitute four interconnected cell culture chambers and one oxygen supply compartment. The microfluidic bioreactor can achieve high cell seeding density of 30–40 million cells/cm^3 with efficient mass transfer, which demonstrated its potential of scalability for EBAL application.

V. CLINICAL EVALUATION

Comprehensive clinical research is carried out in some EBALs. The experiences gained from these clinical trials have provided scientists useful insights into the bioengineering and clinical aspects of the bioreactor designs. These crucial lessons will allow us a step closer to the realization of a clinically validated EBAL that can provide long-term support for patients with liver failure. Section IV will report the latest clinical trials of several EBALs.

A. HepatAssist

HepatAssist, now renamed HepaMate, is one of the EBALs that have been most clinically investigated.[142] HepatAssist was extensively evaluated by phase I clinical trial. The clinical research achievement of HepatAssits was seen as a milestone in the area of EBAL R&D. The bioreactor of HepatAssit is based on the hollow fiber configuration with $(5-7)\times 10^9$ cryopreserved primary porcine hepatocytes attached on microcarrier culturing in its extracapillary space. Plasma separated from patient's blood passes a charcoal absorber and an oxygenator before filtering through a 0.2um thickness membrane to reach the cells for biological mass exchange. The detoxified plasma will be combined with blood cells and return to the patient.

In an uncontrolled clinical study of 39 ALF patients treated with HepatAssist,[143] 32 patients successfully bridged to liver transplant, 6 patients had spontaneous recovery through HepatAssist therapy, of whom 5 were ALF caused by acetaminophen overdose and the 1 month survival rate was 90%. In another controlled clinical study,[144] 13 patients with stage III or IV hepatic encephalopathy, which needs emergency liver transplant, received HepatAssist treatment. 10 of them successfully completed liver transplant in the 9–110 h, 2 had spontaneous recovery, and 1 moved to emergency liver transplant before HepatAssist treatment. The neuropsychological symptom of the patients receiving treatment was significantly improved, with the Glasgow Coma Scale score from 6.5 ± 3.7 down to 9.6 ± 4.4, the bilirubin and transaminase levels decreased significantly. 1 year survival rate was 80%. Although therapeutic complications such as transient hemodynamic instability and bleeding occurred in some patients, retroviral infection, immune rejection, and the other serious complications did not happen. The beneficial effects shown in the above studies encouraged HepatAs-

sist to further evaluate its clinical potential. For this purpose, Demetriou *et al.*[145] reported a large randomized, controlled multicenter trial with a total of 171 patients with fulminant/subfulminant liver failure and primary nonfunction following liver transplantation. In the study, 86 cases received standard medical treatment (SMT), and 85 patients received the treatment of HepatAssist. The results showed that although the plasma bilirubin level of the HepatAssist group was significantly lower than the SMT group, and survival in fulminant/subfulminant liver failure patients of the HepatAssist group was significantly higher than the SMT group (70% versus 37%), for the entire patient group, there was no significant difference in the 30-day survival of HepatAssist versus SMT (71% versus 62%). The main reasons for such results may include the following factors: (1) the impact of liver transplant. There were 55% of the 171 patients who received liver transplant in the study, although it took, respectively, 5 and 3 days for the EBAL and SMT groups before moving to the operation, the survival of the ALF patients was dominantly determined by liver transplantation; (2) the difference in the etiology and severity of ALF patients included in the study. The diversity in the disease itself will have an important impact on the clinical outcome; (3) the utmost complexity of ALF interventions, in addition of the significant variation in the standard of therapy among the treatment centers, makes it hard to uniformly evaluate the efficacy of the individual treatments; (4) the number of patients included in such complicated clinical research was not statistically enough; (5) the exact mechanism of HepatAssist's effect to ALF patients was still unclear. The outcome of the study provides the successors with precious reference.

B. Extracorporeal liver assist device

Extracorporeal liver assist device (ELAD), developed by Sussman *et al.*,[146] is another hollow fiber bioreactor based EBAL. The same as HepatAssist systems, it has completed the controlled clinical trials. The cell source charged in ELAD is the human cell line, C3A. About 200 g of C3A cells are used in its modified dialysis cartridge. The cells are separated from patient plasma by the 75KD capillary membrane.

The first human application of ELAD was for a female patient with liver failure.[147] After a period of 6 days of treatment, both neurological symptoms and clinical parameters were significantly improved, and the treatment was suspended. Unfortunately, the patient died of sepsis a few days later. In its pilot clinical study, ELAD had its short-term safety and metabolic support efficacy tested through the treatment of ten patients with ALF and one with primary nonfunction (PNF).[146] In the study, clinical symptoms of ten patients were improved; six patients successfully completed or avoided liver transplant; and medical complications such as hemodynamic instability, complement system activation, or vital organ function failure did not happen.

In the following pilot controlled clinical study of ELAD, Ellis *et al.*[111] reported that a total of 24 ALF patients divided into two groups, the one with the expectation of potential recovery and another one with critical requirement for liver transplant, randomly received EBAL treatment together with SMT, or the sole SMT control. In the group with recovery potential, the survival for EBAL treatment versus the control was 78% versus 75%, while in another group, it was 33% versus 25%. There were no statistical differences between the two treatments, and the ammonia and bilirubin levels in patients receiving ELAD hemoperfusion were 8% and 20% higher than those before treatment. Therefore, its efficacy still needs further validation.

To further enhance the safety and efficacy of ELAD, Millis *et al.*[148] modified its bioreactor in the following aspects: (1) to promote the mass exchange between the plasma and the cells, the semipermeable membrane pore size was increased from 70KD to 120KD, and the perfusion rate of 150–200 ml/min was changed into 500 ml/min; (2) to increase the cell mass from 200 to 400 g; (3) to set the online dynamic monitoring of oxygen and glucose consumption in the bioreactor in order to understand the metabolic activity of the liver cells; (4) to add an extra filter in the circulatory system to prevent the cell leakage into patient; and (5) to amend the oxygenation device in the system for further improvement of the oxygen level in the bioreactor. With these improvements, C3A cells in the ELAD system can maintain effective metabolic activity even after 12–107 h of treatment. With the modified system support, five ALF patients successfully completed their liver transplant operation, and the 30-day survival was 80%. Neither the improvement of patients' biochemical parameters nor obvious adverse reaction had been observed. Now ELAD is in its clinical trials for acute-on-chronic patients in China and United States, its therapeutic effectiveness remains to be further tested.[2]

C. AMC-bioartificial liver

AMC-bioartificial liver (AMC-BAL), developed by Flendrig *et al.*[149] in the Academic Medical Center of the University of Amsterdam, has its bioreactor uniquely designed of two sheets of spirally wound nonwoven hydrophilic polyester scaffold with 1×10^{10} porcine primary hepatocyte aggregates growing on it, and an integrated hydrophobic hollow fiber between the two sheets to homogeneously supply oxygen and remove CO_2. When plasma is perfused through the extrafiber space, it will directly contact the hepatocytes, which can prevent membrane from fouling block during the process of mass exchange, and be beneficial for oxygenation in the bioreactor. Therefore the efficiency of mass exchange was significantly improved in the AMC-BAL bioreactor than the other hollow fiber bioreactors.

AMC-BAL began its phase I clinical trial in Italy.[114] In the study, seven cases of coma grade III or IV ALF patients received 8–35 h of treatment with AMC-BAL. All patients had improved neurological function, and the blood concentrations of bilirubin and ammonia decreased by 31% and 44%. Six cases were successfully bridged to liver transplant, one underwent liver function recovery after two treatments, without the need of liver transplant. Except for two cases of transient hypotension, no other adverse reactions occurred.

However, since then, many European countries set up legal constraints for xenotransplantation. AMC-BAL group had to suspend the clinical trial research due to the use of porcine hepatocytes, and dedicated themselves to the optimization of bioreactor oxygenation and rheological design as well as the development of human liver cell source.[150]

Although clinical studies showed that AMC-BAL had effects on the survival of ALF patients, but there remained issues to be addressed such as inadequate perfusion of oxygen and fluid inside the bioreactor, which leads to the function of the liver cells dropped dramatically to 25% after 3 days of culture.[151] With further optimization, dead space in the bioreactor was reduced so that the biofunction of liver cells was kept at 80%–90% of day 1 after 3 day cultivation.[152] In addition, Mareels *et al.*[153] reported their application of rheological numerical model for the optimization the distribution of oxygen in the AMC bioreactor. However, it has yet reported the clinical studies with optimized bioreactor AMC. Therefore its safety and efficacy for the ultimate clinical application remain waiting for the large, randomized, controlled clinical trials.

D. Excorp medical bioartificial liver support system

The Excorp medical bioartificial liver support system (BLSS), developed by the University of Pittsburgh (McGowan Institute), has the hollow fiber bioreactor cartridge loaded with 70–100 g of porcine primary hepatocytes mixed with collagen gel.[154] The whole blood gets oxygenated before entering into the bioreactor, where it exchanges mass with liver cells through a semipermeable membrane (100KD). The first case of treatment with BLSS is a 4l-year-old female patient with ALF.[155] After treatment, the patient had the concentrations of ammonia, lactate, and total bilirubin decreased, coagulation function and clinical symptoms improved, and eventually removed off the BLSS treatment. There was another study that reported four cases with ALF treated with BLSS.[112] All the four patients could tolerate the treatment well, and the average levels of ammonia and total bilirubin levels were lower than before treatment by 33% and 6%. During the hemoperfusion, except for the hypoglycemia of four patients at the start phase, and the low blood pressure of one patient that was corrected promptly, there were no other serious complications. And there was no PERV infection detected in all the patients in 1 year after the treatment.

E. Modular extracorporeal liver support

Modular extracorporeal liver support (MELS), developed by the team led by Sauer IM,[115,156,157] has an integrative system including the bioreactor unit, the CellModule charged with primary hepatocytes (from porcine or human), and the detoxication unit, the DetoxModule based on albumin dialysis. In the bioreactor, there is a 3D interwoven fiber matrix by two groups of hydrophilic material and one group of hydrophobic material. The hepatocytes are immobilized on it. The plasma passes through one group of the hydrophilic fiber and contacts with the cells cultured in the extracavity space, then flows out by another group of hydophilic fiber. The hydrophobic fiber supplies oxygen and removes CO_2. The bioreactor has high performance of mass exchange so that it can load 500–600 g of liver cells. In addition, MELS is the only one that uses the primary human cells harvested from the discarded liver tissue or organ in its system.[158]

In one of its phase I clinical studies,[159] MELS loaded with primary porcine hepatocytes was used for the treatment of eight patients with ALF of different etiology for 8–46 h, all patients tolerated the treatment well and were successfully bridged to the liver transplant with 100% of 3 year survival. No adverse events associated with the treatment were observed. No PERV infection was discovered in the patients.

In another clinical trial of MELS using human primary hepatocytes, the bioreactor was charged with human cells harvested from discarded grafts, and was integrated to the albumin-dialysis detoxication unit. After 7–144 h treatment, the neurological symptoms and blood coagulation function of all the eight patients were improved without adverse complications.[158]

F. Radial-flow bioreactor bioartificial liver

Radial-flow bioreactor (RFB) bioartificial liver, developed by the University of Ferrara, Morsiani *et al.*,[160] has the bioreactor in which the afferent fluid perfuses from the center to the periphery while passing through the cells growing in the apartment. 200–230 g freshly isolated porcine hepatocytes are charged in the bioreactor and seeded on the polyester mesh, which is sandwiched between two layers of polyester sheets to prevent cell leakage.

In the pilot phase I clinical trial of RFB bioartificial liver,[127] seven ALF patients with hepatic coma grade II to IV received 6–24 h of treatment. Six of them were successfully bridged to liver transplant: The neurological symptoms and coagulation function were improved; the concentration levels of blood ammonia and bilirubin level decreased by 33% and 11%. No complications such as hemodynamic instability, PERV infection, and immune rejection were observed. All the above results illustrate the clinical potential of the RFB bioartificial liver.

With the development of more than 20 years, EBAL has shown its effect in the preclinical trials of significant improvement of the survival of ALF animal models.[161–164] However, how to rationally design and perform the evaluation of the effectiveness of EBAL treatment remains a crucial issue in the clinical trials. Currently the survival of patients is the most commonly adopted indicator for the efficacy evaluation of EBAL, but once possible, according to the standard therapy, the ALF patients meeting the inclusion criteria of the clinical trials will move to undergo liver transplant only after a short term of EBAL treatment, and the survival is dominantly determined by the surgery effect; thus it is difficult to identify whether the survival improvement is due to EBAL or liver transplantation. The other important indicators for efficacy evaluation of EBAL, such as blood concentration of ammonia and bilirubin, or neurological symptoms, most of them can be influenced by both the biological and the artifi-

cial parts of the EBAL system; therefore how to discriminate the difference of their roles in the EBAL performance is worthy of more consideration.

There are lots of diversities in the etiology and severity of the ALF or ACLF. Considering to such complexity, how to properly individualize the EBAL treatment for different patients, mainly including indications, contraindications, treatment time course, frequency, interval, etc., can also have a serious impact on the final therapy results.

VI. CONCLUSION

In general, due to the complexity of both the treated subject and the system itself, it remains enormously challenging to extensively carry out the large, randomized controlled, multicenter clinical trials for such an advanced therapy until now. For the purpose of clinical application, the current requirements for the EBAL bioreactor design can be summarized as follows: (1) To achieve highly efficient two-way mass exchange between the plasma and the liver cells to meet the needs for maintenance of liver cell metabolism, detoxification, and synthetic functions.[148] (2) To simulate the *in vivo* microenvironment of liver tissue and provide consistent and sufficient support for long-term maintenance of liver cell function and viability, by (a) the improvement of the oxygen supply by independent channels for oxygen supply, amendment of extra oxygenators to the system;[148] or expansion the distribution of oxygen;[152] the addition of the oxygen carriers such as calf red blood cells,[165] perfluorocarbons;[166,167] (b) Optimization of the bioreactor microenvironment, by mimicking the oxygen and nutrient concentration gradient of liver tissue *in vivo*, which may be helpful for the liver cells to re-establish their performance before isolation;[168] (c) improvement of the technology of hepatocyte isolation and cultivation, such as coculture with nonparachymal cells to enhance hepatocyte function; investigation of the advanced material and construct of the supportive scaffold; (3) to facilitate dynamic monitoring the changes of function and biochemical parameters within the bioreactor;[148] and (4) to be easy for cryopreservation, transport, and assembly to fully suit the clinical practice.

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