(54) Title: LIVER ASSIST SYSTEM BASED ON HOLLOW FIBER CARTRIDGES OR ROTATING BIOREACTOR

(57) Abstract: A liver assist system includes (a) a cross-flow configured hollow fiber microfiltration cartridge or (b) a rotating bioreactor, loaded with viable hepatocytes and at least one oxygen source to promote hepatocyte viability. The rotating bioreactor liver assist system may also be fitted with an oxygen source(s) adequate to promote long-term hepatocyte viability, and is optionally fitted with a hollow fiber microfiltration cartridge serving as a filter to prevent the hepatocyte escape from the bioreactor into the subject. Each system is used in a method to metabolize and/or detoxify molecules found in blood, plasma, or other perfusable fluid. The system provides restorative or substitute liver function ex vivo in subjects with liver insufficiency, those in imminent risk of liver insufficiency, or those awaiting liver transplantation. Hepatocytes maintained in this system have the functional attributes of intact healthy liver, manifest by conversion of ammonia to urea and various drugs to their respective metabolites.
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LIVER ASSIST SYSTEM BASED ON HOLLOW FIBER CARTRIDGES OR ROTATING BIOREACTOR

BACKGROUND OF THE INVENTION

Field of the Invention

[0001] The present invention in the field of biology and medicine provides two configurations for a novel liver assist system based on (1) fluid perfusion through a cross-flow configured hollow fiber microfiltration cartridge (HFMC) loaded with viable hepatocytes; and (2) freely suspended viable hepatocytes in a rotating bioreactor.

Description of the Background Art


[0003] Acute liver failure (hepatic failure) presents clinically in either the subacute form, which follows a slow course of hepatic degeneration, or fulminant hepatic failure (FHF), which is rapid and gives rise to hepatocellular dysfunction including severe hepatic encephalopathy (Bernau, J. et al., (1986) Sem. Liv. Dis. 6:97-106; Yanda, R.J., (1988) West. J. Med. 149:586-591; Katelaris, P.H. et al., (1989) Med. Clinics N. Amer. 73:955-970). FHF is generally caused by viral hepatitis, drug reactions or poisoning. In the absence of liver transplantation, the survival rate is about 20% but can vary dramatically (from 6 to 79%) depending on age of the subject and etiology of the disease (Bernau et al., supra; Yanda, supra; Katelaris et al., supra).

[0004] The clinical course of FHF is influenced by the onset of multi-organ failure and mortality increases with the onset of severe clotting abnormalities due to a decrease in coagulation factors, renal failure, pulmonary edema or cardiovascular collapse (Bihari, D.J. et al., (1986) Sem. Liv. Dis. 6:119-128). The development of multi-organ failure is the result of circulating toxins such as mercaptans, phenols, fatty acids, ammonia and molecules which increase vascular permeability (Bernau et al., supra; Yanda, supra; Katelaris et al., supra). Other physiological consequences include decreased blood levels of sodium, potassium, calcium, magnesium and phosphate (Wu J-S, et al., (1988) J Formosan Med. Assoc. 87: 944-947).

[0005] The most severe consequence of this increased permeability is seen in the brain. Toxins cross the blood-brain barrier and produce toxic effects which may lead to vasogenic or cytogenic coma (Bassett, D.J. et al. (1987) Gastroenterology 93:1069-1077; Bernau et al., supra). The final step in this

[0006] Orthotopic liver transplantation is an effective treatment for a wide variety of conditions that destroy hepatic function ranging from genetic defects to tumors (Starzl, T.E. et al., (1989) *N. Engl. J. Med.* 321:1014-1022). Many have attempted to develop “artificial livers” or liver assist systems to keep subjects alive and neurologically intact until their livers either spontaneously regenerated or were replaced by orthotopic liver transplantation. “Artificial livers” are useful in prolonging survival and supporting subjects waiting for a transplant or during a period of functional recovery following transplantation; including cases where primary grafts do not function (Rozga, J, et al., (1995) *ASAIO J* 41: 831-837; Khalili, TM, et al., (2001) *Artificial Organs* 25: 566-570).

[0007] Liver assist systems and techniques, which date back to the late 1950’s, generally were based on the principle of blood detoxification of small dialyzable molecules and toxins that can cause liver failure or that are endogenously produced and elevated in subjects suffering from liver insufficiency (Arkadopolous et al., (1998) *Intl J of Artificial Organs* 21(12): 781-787). For example, using a hemodialysis based artificial liver system, Kiley, JE, et al., (1958) *N Engl J Med* 259:1156-1161, reported temporary reduction in blood-ammonia concentrations of five acute liver failures.

[0008] Liver assist systems utilizing active liver tissue preparations or isolated viable hepatocytes that substitute for defective liver function; including detoxification and biotransformation, trace back to 1956 when Sorrentino et al., (1956) *Chir Patol Sper* 4:1401-1414, demonstrated that liver tissue homogenates could convert ammonia into urea and metabolize salicylic acid, barbituric acid, and ketone bodies. These authors coined the term “artificial liver.”

Ex vivo whole liver perfusion, introduced by Eiseman et al. (1965) *Ann Surg* 162: 329-345, has been used to treat over 100 subjects and later adapted for use with whole livers obtained from human cadavers, pigs, baboons, dogs, and calves (Abouna, G.M., *et al.* (1973) *Surgery* 71:537-547). Fox *et al.*, (1993) *Am J Gastroenterol* 88:1876-1981, treated three subjects by ex vivo perfusion through isolated human livers which were unsuitable for transplantation. Despite improvements, these extracorporeal perfusions were unsuccessful, with survival rates of only 20%.

Clinical usefulness of xenogeneic whole liver perfusion is limited by its technical complexity and the short duration (2 to 6 hours) of liver function due to natural xenonabodies causing hyperacute rejection. Although hyperacute rejection does not occur with "concordant" livers harvested from non-human primates or human cadavers, large primates are scarce and expensive.

Since the 1950's many investigators designed liver assist systems utilizing isolated or cultured liver cells and tissue slices placed in a variety of perfusion bioreactors (Arkadopoulos *et al.*, *supra*). U.S. Patent No. 5,866,420 to Talbot *et al.* discloses an artificial liver support system containing a 1 of normal pluripotent parenchymal hepatocytes and feeder cells derived from a primary culture of pig epiblast cells. This system includes cells layered onto glass plates which serve as a conduit through which fluid perfuses and contacts the layered cells. Still others, most notably (Brunner *et al.*, (1979) *Artif Organs* 3:27-30) employed cellular components and isolated immobilized liver enzymes. Soyer *et al.*, *supra*, designed a liver slice-based system while Eiseman *et al.*, (1976) *Surg Gynecol Obst* 142:21-28, tested isolated hepatocytes in several devices, including a centrifuge, a dialyzer and a perfusion chamber. Eiseman and colleagues also studied membrane hemodialysis in which a suspension of isolated hepatocytes circulated against blood removed from anhepatic pigs. In these devices, liver cells were cultivated on collagen-coated borosilicate glass plates. In most instances, however, the level of detoxification and liver function provided by the above liver assist systems was not significant.

Clinical experience with liver support devices utilizing biological components has been limited to those discussed above. Sussman *et al.*, (1993) *ASAIO Trans* 17: 27-30 reported a bioartificial liver system that relied upon hemoperfusion through a hollow fiber bioreactor containing C3A cells derived from liver tumor (hepatoblastoma) and obtained a patent on an extracorporeal liver assist device using the same (U.S. Patent No. 5,368,555 to Sussman *et al.*) However, the effectiveness of C3A cells and their use in the extracorporeal liver assist devices remains uncertain since there is no evidence that C3A cells maintain hepatocyte-specific functions at the level of primary human hepatocytes (Arkadopolous *et al.*, *supra*). Results of a randomized
controlled clinical trial at King's College Hospital, London, UK, showed no evidence of liver-specific support and no benefits in subjects with FHF. In addition, the use of hepatoma-derived cells to treat severely immunosuppressed subjects raises biosafety issues.

[0014] There exists a need for ex vivo liver assist systems that employ viable and safe liver cells with long lasting, differentiated metabolic functions capable of detoxifying small molecules from plasma, serum, or other body fluid. A suitable ex vivo liver assist system is one in which functional hepatocytes could either proliferate (generally not possible) or survive in a viable, functional state long enough to provide adequate substitutive or restorative liver function to subjects with total or partial liver insufficiency.

[0015] U.S. Patent No. 5,712,154 to Mullon et al., discloses a cell culture unit comprising a liver assist system composed of a dual fiber filter having liquid and gas ports. The dual fibers have extracapillary and intracapillary spaces wherein a cell culture of hepatocytes is situated in the extracapillary space and fluid and gas is perfused through intracapillary space. In this document, however, the hollow fiber filter employed is connected in a parallel-flow (axial-flow) configuration. This document discloses use of cryopreserved hepatocytes that have an initial viability of between about 80% and 99% and retain a significant proportion of the metabolic activity of fresh hepatocytes.

Culture of Hepatocytes and Aggregate/Spheroid Formation


[0017] Landry, J. et al., (1985) J. Cell Biol. 101: 914-923 described the spontaneous formation of spheroidal aggregates when primary rat liver cells were incubated on a non-adherent plastic dish. Three distinct cell morphologies were noted: surface monolayer cells; hepatocytes grouped as islands with deposition of extracellular matrix components; and structures resembling bile ducts. Tong, JZ, et al., (1990) Exp. Cell Res. 189: 87-92, studied multicellular spheroids formed from newborn rat liver cells and detected secretion of liver proteins, specifically albumin and transferrin, for up to 60 days when the culture medium was supplemented with dexamethasone, glucagon, insulin, and epidermal growth factor. These investigators (Tong, JZ, et al., (1992) Exp. Cell Res. 200: 326-332) also observed maintenance of liver-specific functions in spheroid cultures of adult rat hepatocytes, demonstrating metabolism of lidocaine by the cytochrome P450 (CYP) enzyme 3A2, for up to 14 days. CYP 1A1 was strongly induced by methylcholanthrene, remaining constant for up to 22 days.
One of the present inventors and his colleagues (Li, AP, et al., In Vitro Cell. Dev. Biol. 28A: 673-677 (1992a)) was the first to report spheroid formation by human hepatocytes. Their simple, yet proven, method involved seeding 5 x 10^4 hepatocytes in culture medium on a 100-mm plastic dish and “shaking” at 50 rpm overnight. This method caused 90% of the cells to aggregate in the form of spheroids, which possessed many of the morphological characteristics of intact liver.

Li et al. (U.S. Pat 5,270,192) disclosed a hepatocyte bioreactor in which hepatocytes or aggregates were entrapped inside a matrix of glass beads. U.S. Pat. 5,624,839 disclosed hepatocyte spheroid formation promoted by lipid-bound glycosaminoglycan.

Sajiki et al., (2000) ASAIO J. 46:49-55 disclosed a bio-artificial liver assist system in which porcine hepatocytes were loaded into the intracapillary space of hollow fibers of a hemodialyzer. The hepatocytes formed rod-shaped cell aggregates similar to hepatocyte spheroids. Fluid flowed into and out of the cartridge at an angle perpendicular to the hollow fiber filters, i.e., ingress and egress was through the extracapillary space. In this configuration, dialyzable molecules were detoxified by diffusion across the fiber walls into the intracapillary space where they contacted hepatocytes.

Of these documents that disclose hepatocyte loaded “artificial liver” devices or systems, none describe or suggest the superior detoxification properties of a liver assist system having hepatocytes loaded into the extracapillary space of an HFMC where the HFMC is coupled to the system in a trans or cross-flow configuration as disclosed below (see also Figs. 2A, 2B, and 4). In addition, none of these documents describe or suggest the use of freely suspended cells in a rotating bioreactor such as a rotating wall vessel.

Rotating Wall Culture Vessels

Rotating wall vessels or RWVs are a class of rotating bioreactors developed by and for the U.S. National Aeronautic and Space Administration (NASA) beginning in about 1990 that were designed for growth of animal cells in suspension culture in a quiescent environment that simulates microgravity. RWVs were first described in a number of U.S. patents (5,026,650; 5,153,131; 5,153,133) assigned to NASA, and in several additional U.S. patents (5,437,998; 5,665,594; 5,702,941) assigned to Synthecon, Inc., a contractor and licensee of NASA). Other references describe the same principle as the RWV, i.e., horizontal rotation for mixing or suspending cells in culture medium. With the exception of Ingram et al. (U.S. Pat. 5,523,228), however, these patents do not disclose the culture of freely suspended cells. For example, according to Rhodes et al. (U.S. Pat. 5,104,802), cells are confined inside a hollow fiber device rotating with the culture vessel.
U.S. Patent No. 6,117,674 described a process for propagating a pathogen in a three-dimensional
tissue culture mass in RWV culture.

[0023] Rotating wall vessels or RWVs have proven beneficial to the cultivation of many cell
types for tissue engineering applications. Unlike conventional vessels, an RWV accommodates
three-dimensional (3D) assembly and co-location of dissimilar cell types in a gently mixed
environment. The result is more extensive 3D growth with increased cell-cell and cell-matrix
interactions and cellular differentiation that more closely resembles organized living tissue
(Spauding, GF, et al., (1993) J. Cell. Biochem. 51:249-251). These properties of RWVs have been
exploited to grow and study primary cells from various normal tissues (Goodwin, TJ, et al., (1993)
385) and cells from tumors. For example, aggregates of human prostate tumor cells were more
differentiated in terms of their growth, morphology, and cytoskeletal protein expression when
cultured in an RWV compared to “control” tumor cells grown in conventional spinner flasks or static
Vitro Cell. Dev. Biol. 35: 501-509) described the culture of human hepatocytes in an RWV,
primarily providing morphological descriptions, though this document disclosed continuous albumin
secretion and urea nitrogen production over a period of 20 days.

SUMMARY OF THE INVENTION

[0024] This application incorporates by reference in its entirety Silber et al.,
PCT/US03/05368 entitled “Hepatocyte Bioreactor System for Long Term Culture of Functional
Hepatocyte Spheroids (having a priority date of 26 February 2002, having common ownership).

[0025] The present invention provides a liver assist system for metabolizing one or more
compounds in a perfused fluid. In one configuration, the liver assist system includes an HFMC
loaded with viable mammalian hepatocytes and coupled to the other system components in a cross or
trans-flow configuration. In a second configuration, the liver assist system includes (1) a rotating
bioreactor, preferably a perfusion rotating wall vessel (PRWV), loaded with viable mammalian
hepatocytes, (2) an oxygenation source, connected to the rotating bioreactor that supplies sufficient
oxygen pressure to maintain hepatocyte viability, and (3) a means for perfusing fluid through the
system.

[0026] In the preferred embodiment of the first configuration, the liver assist system
comprises a HFMC having an entry port into an extracapillary space with tubing coupled to the entry
port directing the ingress of fluid into the cartridge. The intracapillary space is defined by hollow
fibers separated from the extracapillary space by membrane walls with selective permeability. The
cartridge also has an exit port from the intracapillary space with tubing directing egress therefrom. Viable hepatocytes, which are preferably human or porcine, are loaded into the extracapillary space along with a suitable suspension fluid, e.g., culture medium. The cartridge is coupled to the liver assist system in a cross-flow configuration so that when the system is placed into operation, fluid enters the extracapillary space via an entry port, contacts said hepatocytes, traverses said membrane walls, and exits the cartridge from the intracapillary space via an exit port. A peristaltic pump impels the perfusion of fluid through said system, into and out from the cartridge. To maintain hepatocyte viability, a perfusion oxygenator provides sufficient oxygen concentration to the fluid in which the hepatocytes are situated.

[0027] In the preferred embodiment of the second configuration, the liver assist system comprises a perfusion rotating wall vessel (PRWV) with tubing coupled to the entry port directing the ingress of fluid into the vessel. Viable hepatocytes, which are preferably human or porcine, are loaded into the vessel along with a suitable suspension fluid. To maintain hepatocyte viability, a perfusion oxygenator provides sufficient oxygen concentration to the circulating fluid in which the hepatocytes are situated. A filter, preferably a HFMC, is incorporated downstream of the PRWV to keep hepatocytes from escaping the liver assist system circuit. When the system is placed into operation, a peristaltic pump impels fluid from its source through tubing to an oxygenator, then into the PRWV via an entry port where the fluid contacts the hepatocytes, exits the PRWV through an exit port, and finally moves through a hollow fiber filter.

[0028] The invention, in either of two configurations, provides a liver assist system suitable to provide substitutive liver function to a subject with total or partial liver insufficiency and a method for achieving the same. A subject is preferably connected to one of the above described systems via a venous connection and the subject’s whole blood or plasma is perfused through the system where it contacts the hepatocytes and is treated thereby. Treated whole blood or plasma is then returned to the subject via another venous connection.

[0029] In one embodiment the liver function being substituted or restored is the detoxification of a toxic compound like ammonia or some other endogenously produced metabolite.

[0030] In another embodiment, the liver function being substituted or restored is cytochrome P450 enzyme activity which is responsible for metabolism of compounds like acetaminophen, lidocaine, phenacetin, chloroxazone, 7-hydroxycoumarin, 7-ethoxycoumarin, dextromethorphan, S-Mephenytoin, midazolam, testosterone, tolbutamide, and warfarin. In yet another embodiment, the liver function being substituted or restored is the production of plasma proteins, the formation of
blood coagulation factors, the elimination of aromatic amino acids, the conjugation of bilirubin, glucose metabolism, or heme metabolism.

[0031] The hepatocytes loaded into the liver assist system can be single cells or aggregated hepatocyte, for example in the form of spheroids.

[0032] The fluid can be whole blood, plasma, or serum. Preferably, whole blood is removed from a subject and passed through a plasmapheresis apparatus to separate red blood cells from plasma. The plasma is then pumped to an oxygenator and then on to the device containing the hepatocytes.

[0033] Also provided is a method of metabolizing or detoxifying a compound in a fluid, e.g., whole blood, plasma, or serum, by perfusing the fluid through the HFMC-based or the rotating bioreactor-based liver assist system as described above comprising the steps of (a) perfusing the fluid through the liver assist system; and (b) causing the fluid to contact the viable mammalian hepatocytes in the cartridge under conditions wherein the hepatocytes metabolize or detoxify the one or more compounds. The fluid is preferably from a human. The preferred perfusing means is a peristaltic pump operatively coupled to the liver assist system which actuates the perfusing. In this process, the hepatocytes preferably exhibit at least about 10% of the cytochrome P450 enzymatic activity of a similar number of hepatocyte in a healthy liver in vivo. The metabolizing or detoxifying is generally performed by a cytochrome P450 enzyme in the hepatocytes. The compounds metabolized or detoxified include those listed above.

[0034] In the above process the hepatocytes may be single cells or are in the form of aggregates, optionally in the form of spheroids. The hepatocytes are preferably of porcine, canine, rabbit, murine or primate origin, most preferably human.

[0035] The invention includes a method for metabolizing or detoxifying one or more compounds in a subject, comprising subjecting the blood or plasma of a subject, preferably a human, in need of such metabolizing or detoxifying to the process described above. The subject may be one who suffers from total or partial liver insufficiency. In this method, the connecting of the system is preferably by a venous connection to the subject.

[0036] In this method, as above, the hepatocytes may be single cells or in the form of aggregates, such as spheroids. The method may employ murine, rabbit, canine, porcine or primate hepatocytes, preferably human, in the system.

[0037] In one embodiment of this method, the liver assist system also comprises tubing coupled to the entry port, and the viable mammalian hepatocytes are in a culture medium, the
perfusing means is a peristaltic pump suitable for perfusing fluid into the cartridge, through the system, and out of the cartridge, and, the oxygen providing means is a perfusion oxygenator suitable to maintain the viability of the hepatocytes by providing a sufficient concentration of oxygen to the culture medium in which the hepatocytes are incubated.

[0038] Also included is a method of treating blood or plasma to metabolize or detoxify a compound therein using a HFMC-based liver assist system, comprising the steps of transporting the blood or plasma into the system described above; permitting the blood or plasma to contact the hepatocytes in the extracapillary space of the cartridge under conditions wherein the hepatocytes metabolize or detoxify the compound to produce treated blood or plasma; impelling the treated blood or plasma across the membrane walls into the intracapillary space of the cartridge; and transporting the treated blood or plasma out of the intracapillary space of the system. The above method is used to may provide liver function to a subject in whom liver function is deficient or absent, and comprises connecting the subject to the above liver assist system; perfusing the subject’s blood or plasma through the system, wherein the hepatocytes metabolize or detoxify compounds which had accumulated in the blood or plasma due to the deficient or absent liver function, to produce treated blood or plasma; and returning the treated blood or plasma from the cartridge to the subject.

**BRIEF DESCRIPTION OF THE DRAWINGS**

[0039] **Figures 1A and 1B** are schematic diagrams showing the hollow fiber liver assist system assembled in an axial or parallel-flow configuration. Fig. 1A shows schematically the flow of fluid (arrows) through the assembled system. Fig. 1B shows a cross section (top) and longitudinal section (bottom) of the HFMC indicating the extracapillary and intracapillary spaces and the hepatocytes present in the extracapillary compartment. The longitudinal section illustrates the ingress and egress (broad solid arrows) of fluid in the axial- or parallel-flow configuration. The cross section displays the intracapillary space as a “single” hollow fiber in the center, surrounded by hepatocytes in the extracapillary space.

[0040] **Figures 2A and 2B** are schematic diagrams showing the hollow fiber liver assist system assembled in a trans- or cross-flow configuration. Fig. 2A shows schematically the flow of fluid (arrows) through the assembled system in a schematic diagram. Fig. 2B shows a cross section (top) and longitudinal section (bottom) of the HFMC indicating the extracapillary and intracapillary spaces and the hepatocytes present in the extracapillary compartment. The longitudinal section illustrates the ingress and egress (broad solid arrows) of fluid in the trans- or cross-flow
configuration. The cross section displays the intracapillary space as a "single" hollow fiber in the center, surrounded by hepatocytes in the extracapillary space.

[0041] **Figure 3** is a more detailed schematic flow diagram of the hollow fiber liver assist system assembled in the cross-flow configuration. The components are a hollow fiber membrane cartridge (HFMC) (1) that contains hepatocytes shown as small dark circles, an oxygenation means (2), a perfusion means (here, a pump (3)), and a fluid source (a container (4) or a live subject. Arrows indicate the direction of fluid flow.

[0042] **Figure 4** is a graph showing the detoxification of ammonia after the system is repeatedly "spiked" with ammonia. Following an initial perfusion with 150 μM ammonia, the hollow fiber liver assist system, loaded with $3 \times 10^9$ aggregated Yucatan pig hepatocytes, was challenged with ammonia four more times at one-hour intervals. Also shown in this graph is the accumulation of urea over time. A symbolizes challenge of hepatocytes with NH$_4$Cl.

[0043] **Figure 5** depicts an embodiment of the Rotating Bioreactor liver assist system employing a prior art perfusion rotating wall vessel (PRWV) system as supplied by Synthecon, Inc. The system includes a PRWV (1) as the rotating bioreactor, a relatively low capacity aerator (2), a peristaltic pump (3) and various valves, tubing and connectors. Not shown is an air bubble trap that connects to the Synthecon system at (4).

[0044] **Figure 6** depicts an assembled Rotating Bioreactor liver assist system in a preferred configuration. The system includes the PRWV (1), and a fluid container (5) that is the equivalent of a subject being treated. The system has been modified and improved over that shown in Fig. 5, as described in detail below, by the addition of an external perfused oxygenator (6) to replace the low capacity aerator and a more powerful pump (7). Also shown is an optional in-line hollow fiber filter (8). Also shown are various valves, tubing and connectors. The finer white wires are connections for optional oxygen sensors used to monitor the system.

[0045] **Figure 7** is a schematic and flow diagram of the rotating bioreactor liver assist system assembled in a preferred configuration. The components are a PRWV (1), an oxygenator (2), a pump (3), a fluid source, here a container (4) (and, in its intended operation, a subject), and a hollow fiber filter (5).

**DETAILED DESCRIPTION OF THE INVENTION**

[0046] The present invention is directed to a liver assist system that is designed in two basic configurations. Viable hepatocytes are contained in either a hollow fiber microfiltration cartridge (HFMC) or a rotating bioreactor. In both cases, the system metabolizes compounds, e.g., drugs, and
detoxifies toxic molecules present in a fluid, preferably a body fluid such as blood or plasma. As such, the system is used in a method for treating a subject with liver failure.

[0047] As used herein the term "liver failure" or "hepatic failure" includes any functional liver insufficiency in which at least one liver metabolic pathway is inoperable. These terms include acute liver failure, fulminant hepatitis, fulminant hepatic failure, superacute liver failure, subacute liver failure, hepatic necrosis, virus-induced liver failure, drug-induced liver failure, intoxication-induced liver failure, hepatitis, liver failure due to autoimmune hepatitis, hepatic encephalopathy, jaundice associated liver insufficiency, as well as any other types of hepatic failures known in the art.

[0048] The "subject" of the present invention is a mammal, preferably a human, who is in need of a liver assist system, generally because of total or partial liver insufficiency or in imminent risk thereof. The term also includes those subjects that have recovered normal or near normal liver function after being connected to a liver assist system but who remain connected thereto while awaiting liver transplantation or for other reasons determined by their health care professional.

[0049] "Functional" hepatocytes are hepatocytes of mammalian origin, including murine, canine, rabbit, porcine, or primate, most preferably human, that exhibit metabolic activity characteristic of an intact healthy mammalian liver in vivo. At minimum, "functional" hepatocytes must be capable of metabolizing ammonia to urea.

[0050] When used to describe the actions of a hepatocyte, the term "metabolic activity" or "metabolizing" includes fundamental intermediary cellular metabolism that promotes and maintains hepatocyte viability as well as the normal synthesis of various plasma proteins, e.g., albumin, fibrinogen, various globulins and blood coagulation proteins, e.g., prothrombin and factor VII. Hepatocyte metabolic activity also includes the action on compounds (toxins, drugs) that are abnormally elevated in subjects with liver failure or contribute to symptoms of liver failure. These compounds are typically elevated in samples of blood, plasma, serum, or other body fluids. The metabolic activity of the hepatocytes therefore results in detoxification of compounds that are abnormally elevated in such subjects. Such metabolic activity should include the conversion of ammonia into urea, and preferably also the metabolism of drugs or endogenously produced toxic compounds by one or more enzymes from the cytochrome P450 family of enzymes present in the hepatocytes. Nonlimiting examples are listed in Table 1, below (and discussed in several references, such as Easterbrook J, et al. (2001) Drug Metab. Dispos. 29:141-144). Hepatocyte metabolic activity includes the elimination of aromatic amino acids, the conjugation of bilirubin, glucose metabolism, and heme metabolism.
[0051] As used herein the term “substitutive” or “restorative liver function” means the substitution of or restoration of metabolic activity as described above.

**Operation of the Hollow Fiber Liver Assist System**

[0052] The liver assist system of the present invention is designed to substitute for, or restore, normal liver function in subjects suffering from total or partial liver insufficiency. The system is assembled in a “loop” of interconnected components in order for perfused fluid to be circulated from the subject through the several components and return to the subject following detoxification. The primary detoxification means of the hollow fiber liver assist system is a HFMC loaded with hepatocytes as single cells, aggregated hepatocytes, or aggregated hepatocyte spheroids, capable of metabolizing dialyzable molecules found in perfused plasma, serum, or other body fluid. The subject is connected to the liver assist system by any means known in the art, preferably by a direct venous connection.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Metabolites measured</th>
<th>CYP Enzyme</th>
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</thead>
<tbody>
<tr>
<td>Tolbutamide</td>
<td>4-OH-tolbutamide</td>
<td>2B;2C</td>
</tr>
<tr>
<td>Warfarin</td>
<td>4-OH-warfarin</td>
<td>3A</td>
</tr>
<tr>
<td></td>
<td>6-OH-warfarin</td>
<td>1A;2C</td>
</tr>
<tr>
<td></td>
<td>7-OH-warfarin</td>
<td>2C</td>
</tr>
<tr>
<td>Chlorozoxazine</td>
<td>6-OH-chlorozoxazine</td>
<td>2E;1A</td>
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<td>2D</td>
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<tr>
<td></td>
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<td>3A</td>
</tr>
<tr>
<td></td>
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<td>2D;3A</td>
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<td></td>
<td>a. glucuronide</td>
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<td>2C</td>
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*Phase II metabolism enzymes: GST = glutathione-S-transferase; UDP-GT = uridine diphosphate glucuronyltransferase*

[0053] The HFMC may have at least two ports that permit fluid perfusion therethrough.

Hepatocytes are preferably loaded into the extracapillary spaces of the fiber lumen. In an exemplary
cross-flow configuration (Fig. 2A and 2B), a peristaltic pump, serving as a preferred perfusion means, impels fluid to ingress into the extracapillary space where it directly contacts the viable hepatocytes. The hepatocytes should be functional in that they exhibit metabolic activity comparable to that of healthy liver such that they metabolize ammonia and drugs found in the perfused fluid at least to a level of about 10% of healthy liver, preferably at least 20%, and more preferably at least about 40%.

[0054] HFMCs useful in the present invention may be manufactured from any of a number of polymers as is well-known in the art, for example, polyethylene, polypropylene, polysulfone, polyurethane, etc. The hollow fiber filters typically have lengths of between about 7 cm to about 40 cm preferably from about 20 cm to about 30 cm. Those exemplified herein are 25 cm.

[0055] The diameter of the fibers can range from about 150 μm to about 1.5 mm, preferably from about 200 μm to about 650 μm, and most preferably about 250 μm to about 350 μm.

[0056] Preferably, the pore size of the fibers should be less than about 5 μM and preferably between about 0.1 μM and about 1 μM. The intracapillary volume should be about 80 mL to about 250 mL, and preferably between about 100 mL to about 200 mL. The extracapillary volume should be about 70 mL to about 500 mL, and preferably about 100 mL to about 300 mL. Higher capacity cartridges are also useful so long as they can sustain a suitable pressure gradient to maintain fluid perfusion. Although higher capacity cartridges are preferred, lower capacity laboratory-scale cartridges, with an extracapillary volume of 10 mL to 50 mL are also available from A/G Technologies. Among smaller sized cartridges, there are a range of available fiber diameters (0.25 mm to 1 mm) and fiber surface areas (about 3000 cm² to about 9000 cm²).

[0057] HFMCs can be obtained commercially from sources like A/G Technologies (Amersham BioSciences) or Spectrum Laboratories (Rancho Dominguez, CA). One particular cartridge exemplified herein is Model CFP-6-D-8A from A/G Technology Corp., Needham, MA, which consists of 750 polysulfone fibers with a total fiber surface area of 4100 cm², wherein each fiber has an internal diameter of 0.75 mm, and a pore size of about 0.65 μM.

[0058] HFMCs are selected for their:

(a) permeability to fluid and plasma components, such as proteins
(b) maintenance of the desired system flow rate;
(c) permissiveness to dialysis of small molecules such as ammonia and drugs, and
(d) property of keeping hepatocytes from migrating into the intracapillary space.

[0059] The HFMC can be coupled to the liver assist system using "parallel-flow" (axial-flow) or "cross-flow" (trans-flow) configuration. The fluid dynamics of these configurations are

[0060] In the “parallel-flow” configuration (Fig. 1B), fluid enters an entry port (133a), flows axially through the intracapillary lumen (136) and leaves through an exit port (133b). Components of the fluid pass into the extracapillary space (135) by way of trans-fiber diffusion due to a concentration gradient across the semipermeable membrane of the hollow fibers. Thus, there is no direct contact of the fluid with the hepatocytes (137) in the axial-flow configuration. In this configuration, ports (134a and 134b) are closed or blocked by a stopper, plug, cap, valve or any other blocking or closing means (138).

[0061] In the preferred “cross-flow” configuration (Fig. 2B), fluid enters the extracapillary space (135) via entry port (134a), preferably at an angle approximately perpendicular to the axis of the hollow fibers and directly contacts the hepatocytes (137). In this configuration, a pressure gradient impels the fluid into the intracapillary space of the fibers (136) and, axially, out of the cartridge through an exit port (133b). As pictured, two ports are closed or blocked (138) by a blocking or closing means as described above.

[0062] The mass transport advantages of the preferred “cross-” or trans-flow configuration of the liver assist system over a standard “parallel-” or axial-flow configuration are clearly shown in data from Giorgio et al., (1993) ASAIO J 39: 886-892. For any given flow rate to the cartridge produced by the pump, the transfer of a solute across the hollow fibers is faster in a trans-flow than in an axial-flow configuration. In fact, in the trans-flow configuration, overall transport of glucose and albumin were increased by 398% and 535%, respectively. This is attributed to increased solvent transport driven by a larger pressure gradient between the extracapillary and intracapillary spaces and to the addition of an osmotic pressure gradient, at least for smaller solute molecules such as glucose.

[0063] In the preferred embodiment, flow to the HFMC enters the extracapillary compartment, thereby directly contacting the hepatocytes therein, and also exits from the intracapillary compartment. The clear differences in mass transport between “cross-flow” and “parallel-flow” for the present invention are shown in the examples below. The obvious advantage for using a “cross-flow” configuration for a liver assist system is that the hepatocytes will have better access to the solute components of the blood or plasma and will thus be able to metabolize or detoxify these compounds more efficiently.
[0064] Hepatocytes require oxygenation to maintain viability (Balas, UJ, et al., (1999) *Metabol. Eng.* 1: 49-62). The oxygenation source is preferably a perfusion oxygenator that provides oxygen to hepatocytes contained in the liver assist system, maintaining their viability for a sufficient period, at least about one hour, so that restorative or substitutive liver support can be rendered to a subject. The oxygenation source is preferably coupled to a port on the HFMC so that it is situated in the path (i.e., “in-line”) of the circulating fluid (Fig 2A). Oxygenation sources, for example, an Extracorporeal Membrane Oxygenation (ECMO) oxygenator, an OXY-10™ oxygenator (Unisyn, Hopkinton, MA), or an OX-2™ oxygenator (Living Systems Instrumentation, Burlington, VT) are selected for their ability to deliver oxygen at a sufficient pO₂ to maintain hepatocyte viability for the prescribed period. To prevent oxygen dissipation, tubing, for example, C-flex tubing (Cole Parmer, Vernon Hills, IL), which is approximately 30 times less permeable to oxygen than silicone tubing, is preferred for coupling the oxygenator to the cartridge. The skilled artisan will be able to select other compatible tubing having limited oxygen permeability using only routine experimentation.

[0065] One means for obtaining plasma from a subject is a conventional plasmapheresis device which separates the cells from the fluid fraction of the blood. Examples are a Plasmaphlo AP-05H membrane plasma separator (Asahi Medical, Tokyo, Japan) or a Cell Saver autologous blood recovery system (Haemonetics, Braintree, MA). The separated plasma is then pumped to the HFMC, preferably after passage through an oxygenation means or oxygenator to increase the dissolved oxygen content of the plasma.

[0066] A subject is connected to the liver assist system by any means known in the art but preferably by intravenous catheterization, by which whole blood may be removed, preferably to a plasmapheresis device as noted above. The subject’s blood, plasma (or another body fluid) perfuses through the liver assist system, undergoes detoxification by the hepatocytes in the HFMC and is then returned to the subject through another venous connection.

[0067] The perfusion of plasma (or other fluid) is preferably actuated by a pump, e.g., a peristaltic or positive-displacement pump like the MasterFlex® Console Drive Pump (available from the Cole-Parmer Instrument Company, Vernon Hills, IL) or similar variable-speed drive pump, coupled to the system preferably by tubing, and supplying sufficient pressure to impel fluid through the system. The pump supplies pressure to the liver assist system via tubing that joins the fluid source (container as illustrated in the drawings, or, when used as intended, a subject in need of such treatment) and the HFMC. The pump impels the ingress of fluid into the HFMC where the fluid contacts the viable hepatocytes in the extracapillary space and is detoxified thereby. The pressure supplied by the pump is also sufficient to impel the egress of the fluid from the cartridge and back to
the container or subject. The Masterflex Easy Load pump head, using, for example, a 100 rpm drive and Masterflex L/S 17 tubing, can achieve flow rates of up to 280 mL/min.

Tubing for directing perfusion should be medically suitable and biocompatible so that it does not impart toxic properties to the fluid entering and leaving the subject. The tubing is preferably made of silicone, polypropylene, polyurethane, flexible polyvinyl chloride or thermoplastic rubber, and preferably has a low drag coefficient to achieve substantially unfettered fluid perfusion.

**Operation of the Rotating Bioreactor Liver Assist System**

The liver assist system of the present invention is designed to substitute for, or restore, normal liver function in subjects with liver failure. The system is assembled in a “loop” of interconnected components in order for perfused fluid to be circulated from the subject through the several components and return to the subject following detoxification. The primary detoxification means of this liver assist system is a rotating bioreactor, preferably a PRWV, loaded with viable and functional hepatocytes capable of metabolizing compounds, preferably dialyzable compounds, found in plasma, serum, or other fluid circulated or perfused therethrough. The subject is connected to the liver assist system by any means known in the art but preferably by a direct venous connection.

A preferred bioreactor is a perfusion rotating wall vessel (PRWV) having a total volume of about 250 mL (available from Synthecon, Inc., Houston, TX), filled with fluid and containing at least about $10^9$ hepatocytes, although a broad range of cell concentrations may be used.

The assembled liver assist system, including the PRWV, is shown in Figures 5 and 6, with a flow scheme illustrated in Figure 7. A pump supplies pressure to the liver assist system via tubing that joins the rotating vessel at an entry port. Fluid is impelled into the bioreactor where it contacts the viable hepatocytes and is detoxified thereby. The walls of the PRWV are composed of a nontoxic polymer, preferably polycarbonate (polished to be optically clear) and the vessel endcaps are preferably made of white polyacetyl. The inlet endcap incorporates two sample/injection sites, with either stainless steel valves or disposable syringe ports for sampling and feeding and, for example, a 3/8 inch drain or access port with a Swagelok cap. An 8 μm polycarbonate filter is inserted inside the vessel, covering the outlet endcap. The volume capacity of the vessel is approximately 250 mL. All vessel parts can be autoclaved.

During operation, the PRWV is filled with fluid and rotated about a horizontal axis. The speed of rotation is controlled at a rate fast enough to suspend the cells (or aggregates) without exerting excessive shear forces on the cells or causing excessive collisions with the rotating vessel wall. For operation, the vessel is mounted on a motor shaft. Cells are evenly distributed and semi-

[0073] As previously noted, hepatocytes require oxygenation to maintain viability. A variety of oxygenation sources, such as those described in the previous section, may be used to perform this function. In the Rotating Bioreactor embodiment herein, the perfusion of plasma, serum, or other fluid is preferably actuated by a pump. The same types of pumps and tubing described in the previous section may be used.

[0074] The liver assist system (e.g., Fig. 5, 6 or 7) is preferably connected to the subject such that blood, serum, or other body fluid is perfused (circulated) through the system and detoxified by the hepatocytes in the rotating bioreactor and then returned to the subject through another intravenous connection.

[0075] Below are exemplary embodiments for preparing and using the liver assist system. It is understood by those of skill in the art that the procedures and reagents described may be modified by the user in accordance with conventional procedures and knowledge in the art of cell culture and drug metabolism. For example, volumes, cell numbers, reagent concentrations, times, etc., may be subject to a range of modifications without materially changing the nature of the present invention.

**Hepatocytes**

[0076] Mammalian hepatocytes, preferably primary hepatocytes, are loaded into the HFMC or PRWV of the liver assist system and are responsible for detoxification and metabolism of fluid perfused through the system. (See, for example, Stange, J. et al., (1995) *ASAIO J.* 41: M310-M315). Hepatocytes are selected for their functional and/or their morphological similarity to cells of healthy intact livers. Preferably primate, and most preferably human hepatocytes, are used. Porcine hepatocytes are also useful because of their functional and morphological similarities to human cells (Donato, MT, (1999) *J. Hepatology* 31: 542-549, see also Shito, M., et al., (2001) *Artificial Organs* 25: 571-578, and Borie, DC, et al., (2001) *Transplantation* 72: 393-405). Murine, canine, and rabbit hepatocytes can also be used. The invention is not limited to primary hepatocytes. In one embodiment, immortalized human fetal liver sinusoidal cells are used (e.g., Hering S et al., *Exp Cell Res* 195:1-7 1991). Hepatocytes from long term cell lines, e.g., C3A and HepG2, or other hepatocellular carcinoma line, may also be used provided that these cells are functional as described herein, and are disabled from forming tumors if any cells escape from the system and enter the subject. Use of any immortalized or genetically engineered human hepatocytes yet to be produced or discovered are intended to be within the scope of this invention.
Hepatocytes should be viable and metabolically functional for at least one hour to be suitable for use in the liver assist system. Hepatocyte viability can be extended by first aggregating the cells and inducing spheroid formation using methods disclosed by Silber et al. in commonly-assigned U.S. PCT International Patent Application No. PCT/US03/05638, filed February 26, 2003, herein incorporated by reference in its entirety. “Long term” hepatocyte viability may be any period of time exceeding that which was previously known for hepatocytes, e.g., about 4 days, most preferably at least about 7 days, but can be about 14 days or even more than 30 days.


Rat hepatocytes are commonly derived from male Sprague-Dawley rats weighing 225-250g. Porcine, monkey or ape hepatocytes as well as human hepatocytes prepared from livers obtained from accident victim donors (in the case of humans) or other recently deceased donors in whom liver function is believed to be normal or near normal, are prepared in a similar manner, though volumes of reagents are adjusted accordingly.

Although a typical total cell number is about $10^6$ hepatocytes, and although between about 1 and $5 \times 10^6$ is preferred, a broader range may be used, for example, between about $5 \times 10^8$ and $1 \times 10^{10}$ cells, as long as the selected cell number endows the liver assist system with adequate liver function to fulfill its desired role.

A viable, long term hepatocyte culture can be achieved by perfusing mammalian, such as murine or porcine, liver in vivo to yield single cell hepatocyte preparations. Primary hepatocytes may optionally be cultured for a course of 4 to 5 hours in any suitable commercially available medium. When transferring hepatocytes to the HFMC, they are preferably in an isotonic fluid compatible with plasma in which they are maintained up to and after use (if the HFMC is to be recycled). Cell viability is determined by conventional means, such as hemocytometer counts using a vital stain, e.g., trypan blue.

**Ammonia Metabolism**

Ammonia produced from the metabolism of proteins or from other metabolic pathways is excreted by the body directly by the kidneys or first converted into urea by the liver. Excess ammonia is transported to the liver in the form of glutamine. Glutamine synthase catalyses
the ATP-mediated combination of glutamate and ammonia into glutamine. In the liver, glutamine is reconverted into glutamate by glutaminase, releasing an ammonium ion (NH$_4^+$). Glutamate can be further de-aminated by aminotransferase I into α-ketoglutarate and another NH$_4^+$ ion (Voet, D et al., *Fundamentals of Biochemistry* (1999) John Wiley & Sons, pp. 690-692).

[0083] Free NH$_4^+$ ions enter hepatic mitochondria and are carboxylated into carbamoyl phosphate by carbamoyl phosphate synthase I in the presence of a bicarbonate anion in an ATP-mediated reaction. Ornithine transcarbamylase combines carbamoyl phosphate with ornithine to form citrulline which is transported to hepatocyte cytosol where argininosuccinate synthase catalyzes its reaction with aspartate to form arginosuccinate. Arginosuccinate is subsequently cleaved into fumarate and arginine by argininosuccinate lyase. Arginase cleaves arginine into ornithine, releasing urea which is soluble and non-toxic and easily passed into the urine for excretion. The ornithine is transported back into the hepatic mitochondria by way of an ATP-dependent ornithine transport protein where it reenters the urea pathway (Voet et al. *supra*).

[0084] In some subjects experiencing partial or total liver failure, ammonia metabolism is inhibited or totally disabled leading to ammonia toxicity and hepatic encephalopathy (hepatic coma) (Burton, BK, et al., (2000) *Clin Liver Dis 4*: 815-30). The liver assist system of this invention utilizes viable, metabolically functional hepatocytes that can convert ammonia to urea and can detoxify perfused plasma, serum, or other body fluid from subjects with liver failure or in imminent danger of ammonia toxicity.

**Cytochrome P450s (CYPs)**

[0085] CYPs are a family of enzymes, localized to the cytoplasmic side of the endoplasmic reticulum of hepatocytes that catalyze the oxidation of organic compounds, resulting in increased water solubility which promotes excretion from the cell. CYPs are generally important for processing xenobiotics. See Table 1 above and PCT/US03/05368 for examples of liver CYP enzymes that are responsible for the metabolism and detoxification of various drugs.

[0086] Activity of these enzymes is rapidly lost after hepatocytes are isolated from the liver and placed in conventional primary cultures. This loss is particularly prominent for rat hepatocytes, which lose 80% of their CYP activity in the first 24 hours of culture (Paine, AJ, In: Berry, MN et al. (eds.), *The Hepatocyte Review*, Kluwer Academic Publishers, Netherlands, pp. 411-420, 2000).

[0087] It is preferred that viable hepatocytes loaded into the present liver assist system maintain at least about 10%, preferably at least 20%, and most preferably at least about 40% metabolically active cytochrome P450 enzyme pathways as compared to hepatocytes of normal healthy liver. It is desirable that the total CYP activity in hepatocytes loaded into the HFMC or
PRWV is suitable to provide substitutive or restorative drug detoxification to perfused whole blood, plasma, serum, or other body fluid from a subject whose liver is deficient in one or more of these enzyme pathways.

[0088] In some subjects experiencing partial or total liver failure, drug clearance is impaired or totally disabled which may result in overdose and clinical drug toxicity (Cheng PY, *et al.*, (2001) *Curr Drug Metab* 2:165-183). The present liver assist system aids such subjects because of the presence of viable hepatocytes with metabolically active cytochrome P450 enzyme pathways provides substitutive or restorative drug detoxification to perfused plasma, serum, or other body fluid of a liver-deficient subject.

[0089] Below are exemplary embodiments for preparing and using different embodiments of the present liver assist system. Those of skill in the art will understand that the procedures and reagents described may be modified by the user in accordance with conventional knowledge in the art of cell culture and drug metabolism. For example, volumes, cell numbers, reagent concentrations, times, etc., may be subject to a range of modifications without materially changing the nature of the present invention.

**EXAMPLE I**

**Perfusion Configuration of Hollow Fiber Liver Assist System**

[0090] KHB buffer colored with red dye was used to compare mass transfer rates in a hollow fiber liver assist system configured for parallel flow (Figs. 1A and 1B) versus cross-flow (Figs. 2A and 2B). More rapid mass transfer and system equilibration was observed using the cross-flow configuration (about 20 minutes) versus the parallel-flow configuration (about 80 minutes) as shown in Table 2.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Relative Dye Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cross Flow</td>
</tr>
<tr>
<td>0</td>
<td>0.000</td>
</tr>
<tr>
<td>2</td>
<td>0.000</td>
</tr>
<tr>
<td>4</td>
<td>0.000</td>
</tr>
<tr>
<td>6</td>
<td>0.000</td>
</tr>
<tr>
<td>8</td>
<td>0.000</td>
</tr>
<tr>
<td>10</td>
<td>0.003</td>
</tr>
<tr>
<td>15</td>
<td>0.173</td>
</tr>
<tr>
<td>20</td>
<td>0.304</td>
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<tr>
<td>25</td>
<td>0.372</td>
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<tr>
<td>30</td>
<td>0.363</td>
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<tr>
<td>35</td>
<td>0.358</td>
</tr>
<tr>
<td>45</td>
<td>0.365</td>
</tr>
<tr>
<td>60</td>
<td>0.364</td>
</tr>
<tr>
<td>80</td>
<td></td>
</tr>
</tbody>
</table>
EXAMPLE II

Oxygenation of the Viable Hepatocyte Cultured in a Rotating Bioreactor

[0091] Using the original oxygenator (Fig. 5 (2)) provided by the manufacturer of a rotating bioreactor (Synthecon) as the initial oxygen source, the inventors observed only short term hepatocyte viability. The original oxygenator provided by Synthecon has a coil of thin walled silicone tubing with a surface area of 258 cm² over which oxygen from ambient air can diffuse into the circulating fluid. pO₂ levels were monitored using the i-STAT system (i-STAT Corp., Princeton, NJ) or the O₂-ADPT system (Microelectrodes, Inc.) with voltage signals acquired by laptop computer. A sharp decrease in pO₂ was detected soon after cells were loaded into the rotating bioreactor using the original oxygenator. Cell viability decreased as pO₂ increased. The pO₂ supplied by the original oxygenator (Fig. 5 (2)) was inadequate to support the desired hepatocyte viability.

[0092] Thus, in the case of inadequate oxygenation for the desired hepatocyte viability, oxygenation was supplemented by inclusion of one (or possibly more) external oxygenation means or sources (e.g., Fig. 6 (6)). One such source is an OXY-10 oxygenator (available from Unisyn, Hopkinton, MA) or an OX-2 oxygenator (available from Living Systems Instruments) (Fig. 6 (6)). These oxygenators can be readily connected to an oxygen tank for superior oxygen transfer to the liquid flowing through the liver assist system (Fig. 6 (6)). When 95% oxygen is fed to the OX-2 or OXY-10 oxygenators, the pO₂ can reach up to 700 mm Hg compared to approximately 160 mm Hg using the original Synthecon oxygenator. It should be noted, however, that oxygen losses occur throughout the system. Therefore, to maintain higher oxygen levels, it is also preferred to select tubing with relatively low O₂ permeability; one example is C-flex tubing (Cole Parmer, Vernon Hills, IL), which is approximately 30 times less permeable to O₂ than conventional silicone tubing.

[0093] The pO₂ provided by the oxygen source of the liver assist system should be modulated, if necessary, to sustain optimal hepatocyte viability and metabolic activity (Balis, UJ, et al., 1999) Metabol. Eng. 1: 49-62). Optimal pO₂ levels can be determined using routine skill in the art and can be adjusted to obtain the desired density of viable hepatocytes.

[0094] An oxygen tank having a gauge that permits modulation of pO₂ can also be connected directly to the system as an oxygen source. Other oxygen sources can be fitted for use with the liver assist system as will be apparent to those having ordinary skill in the art and can be configured to fit the liver assist system using routine skill and without undue experimentation.
[0095] Oxygen, for example at a concentration of 100% (v/v) can be perfused through the oxygenator to ensure that the hepatocytes are sufficiently oxygenated. A liver assist system fitted with an external oxygenator maintained viability of a rat hepatocyte suspension loaded into rotating bioreactor for a five hour measuring period. Optionally, the original oxygenator (see Fig. 5(2)) can be omitted (or bypassed) altogether and an external oxygenator of greater capacity used as the exclusive oxygen source for the system (e.g., Fig. 6 (6)).

[0096] Mini pig hepatocytes cultured in KHB and loaded into the rotating bioreactor of the liver assist system fitted solely with the external “supplemental” oxygenator as the oxygen source, remained viable over a five hour measuring period.

EXAMPLE III

A PRWY Liver Assist System Fitted with a Hollow Fiber Microfiltration Cartridge

[0097] In the device described above, hepatocytes showed a tendency to escape from the rotating bioreactor (Fig. 6 (1)) and to perfuse into the container (Fig. 6 (5)) which would be the subject in the invention’s intended operation. To prevent this, a hollow fiber microfiltration cartridge was added to the system as shown in Fig. 6 (8) and Fig. 7 (5) for the purposes of capturing and containing cells that escaped from the bioreactor.

[0098] A hollow fiber microfiltration cartridge that can be fitted to the present liver assist system is preferably selected for its

(a) permeability to fluid and plasma;
(b) ability to maintain the desired system flow rate and presence of sufficient extracapillary fiber space to accommodate between about 10% and about 50% of the cell mass from the rotating bioreactor;
(c) ability to dialyze small molecules such as ammonia and those listed in Table 1; and
(d) ability to exclude hepatocytes that escape from the rotating bioreactor from straying into the container (Fig. 6 (5)) or the subject being treated.

[0099] One such hollow fiber microfiltration cartridge can be, for example, the Model CFP-6-D-8A from A/G Technology Corp., Needham, MA, made of polysulfone fibers and having 750 fibers with a total fiber surface area of 4100 square cm, an internal diameter of 0.75 mm, and a fiber pore size of about 0.65 μM. Preferably, the pore size should be less than about 5 μM and preferably between about 0.1 μM and 1 μM.
The intracapillary volume is about 80 mL to about 250 mL, and preferably between 100 mL and 200 mL. The extracapillary volume is about 60 mL to about 100 mL, and preferably about 100 to about 300 mL. Higher capacity cartridges are also useful so long as a suitable pressure gradient is maintained for perfusion.

Lower capacity laboratory-scale cartridges, with an extracapillary volume of 10 to 50 mL are also available from A/G Technologies, however, higher capacity cartridges are preferred. Among similar sized cartridges, there are a range of available fiber diameters (0.25 to 1 mm) and fiber surface areas (about 3000 to about 9000 square cm).

One purpose of the hollow fiber filter, then, is to keep the “container” (Fig 7 (4) and Fig. 7 (4)), representing the subject being treated, free of straying hepatocytes while allowing protein transfer across the fibers.

The hollow fiber microfiltration cartridge, when used in the rotating bioreactor embodiment (Fig. 6 (8) and Fig. 7 (5)) can be connected to the rotating bioreactor in a “parallel-flow” or a “cross-flow” configuration (MacDonald, JM, et al., (2001) Ann NY Acad Sci 944:334-343).

In the “parallel-flow” configuration, fluid flows axially through the intracapillary fiber lumen space and fluid components have access to the extracapillary space only through diffusion across the semipermeable fiber membrane.

In the preferred “cross-flow” configuration, fluid enters the column directly into extracapillary space, at an angle perpendicular to the hollow fiber bundle. Fluid egress is achieved both by a pressure gradient in the extracapillary space and a trans-fiber diffusion gradient that impels fluid to enter the intracapillary lumen where it leaves axially (Giorgio, TD, et al., (1993) ASAIO J. 39:886-892).

Hepatocytes that escape from the rotating bioreactor are halted from further perfusion and become entrapped in the hollow fiber microfiltration cartridge and are thereby prevented from entering the container/subject.

EXAMPLE IV

Ammonia Detoxification by the Liver Assist System

One important function of the liver is to convert serum ammonia to urea for excretion by the kidney. Normal adult blood ammonia levels in humans range between 10 and 35 μM. A subject in fulminant hepatic failure may present with blood ammonia levels of 155±10 μM (Watanabe, FD, et al., (1997) Ann Surg 225: 484-494). To simulate a subject with blood ammonia

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concentrations characteristic of liver failure, a solution of 150 μM NH₄Cl in KHB was added to the fluid source (container) of the liver assist system. The HFMC was loaded with 1.3 x 10⁹ mini-pig hepatocytes and the ammonium chloride solution was perfused through the system. Ammonia concentrations during “treatment” were monitored using samples from the container. An identical liver assist system was perfused without any hepatocytes loaded in the HFMC. This system served as the control. Ammonia was measured via one or both of the following methods: (1) the Sigma urea nitrogen kit (procedure No. 640), modified for smaller sample volumes and without the urease treatment, and with absorbance measured at 590 nm; or (2) the Ammonia Checker II (Arkray; Kyoto, Japan). As seen in Table 3, the control system had virtually no change in ammonia levels while in the treated system, ammonia concentrations fell to “normal” physiological levels within at least 5 hours.

The ammonia detoxification function was also tested in the Rotating Bioreactor liver assist system. Again, a solution of 150 μM NH₄Cl in KHB was added to the fluid source of the liver assist system. The PRWV was loaded with 3 x 10⁹ mini-pig hepatocytes and the ammonium chloride solution was perfused through the system. Table 4 shows that this system was also capable of ammonia detoxification, with ammonia levels dropping by 2.5-fold after 4 hours. The final ammonia level was not quite as low as that achieved using the Hollow Fiber system (Table 3).

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Control (no hepatocytes)</th>
<th>Treatment (plus hepatocytes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>164</td>
<td>137</td>
</tr>
<tr>
<td>1</td>
<td>159</td>
<td>86</td>
</tr>
<tr>
<td>2</td>
<td>149</td>
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<td>133</td>
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</tr>
<tr>
<td>8</td>
<td>119</td>
<td>31</td>
</tr>
</tbody>
</table>

Table 3. Ammonia Detoxification by Hepatocytes in the Hollow Fiber Liver Assist System

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Ammonia concentration (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>139</td>
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<tr>
<td>1</td>
<td>112</td>
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<td>2</td>
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<td>4</td>
<td>56</td>
</tr>
<tr>
<td>20</td>
<td>59</td>
</tr>
</tbody>
</table>

Table 4. Ammonia Detoxification by Hepatocytes in the Rotating Bioreactor Liver Assist System
EXAMPLE V

Response of the Hollow Fiber Liver Assist System to Ammonia “Challenge”

[0109] The ammonia detoxification properties of the hollow fiber liver assist system was further evaluated by challenging the system loaded with 3 x 10⁹ Yucatan pig hepatocytes with hourly spikes of approximately 150 μmoles of ammonium chloride. The system was sampled each hour, before and after each addition of ammonia. This test was deemed important because in a true clinical situation, a liver assist device would likely be repeatedly challenged with fresh plasma containing toxic levels of various compounds, particularly ammonia. In this experiment, ammonia was measured as detailed above. Urea was measured with a Sigma blood urea nitrogen kit (procedure no. 535), using smaller sample dilutions (1:5) and standards in the range of 10 to 200 μM urea.

[0110] As shown in Figure 4, the liver assist system loaded with hepatocytes was able to detoxify the ammonia after each spike, reducing the concentration by 2.5 to 4-fold each time. Concurrently, there was a steady synthesis of urea to a concentration of 210 μM after 5 hours. In contrast, in the control system without hepatocytes, ammonia accumulated to a level of approximately 500 mM, and no urea was produced.

EXAMPLE VI

Drug Metabolism by the Hollow Fiber Liver Assist System

[0111] The hollow fiber liver assist system demonstrated drug detoxification capability when tested for its ability to metabolize 7-ethoxycoumarin (7-EC). Complete metabolism of this drug is via both Phase I and Phase II pathways, involving the cytochrome P450 enzymes 1A1/2 and 2A6 and further metabolism by the conjugation enzymes glutathione S-transferase and uridine diphosphate glucuronyltransferase. The hollow fiber liver assist system was loaded with Yucatan pig hepatocytes and perfused with 100μM 7-EC/KHB solution. Samples from the system were taken hourly to assay for substrate and metabolites using high performance liquid chromatography (HPLC).

[0112] HPLC analysis was performed on an Agilent (Palo Alto, CA) model 1100 liquid chromatograph fitted with a photodiode array and fluorescence detector. The substrate 7-EC and its metabolites 7-hydroxycoumarin (7-HC); 7-hydroxycoumarin glucuronide (7-HCG); and 7-hydroxycoumarin sulfate (7-HCS)] were separated on a LUNA C18 column (Phenomenex,
Torrance, CA; length 250 mm x diameter 4.6 mm) and detected at a wavelength of 320 nm using a mobile phase gradient.

[0113] The results in Table 5 demonstrate production of all three metabolites. In particular, 7-HCG was produced in large amounts, with a four-fold increase by the end of sampling at 21 hours. A separate “control” liver assist system with no hepatocytes loaded was run simultaneously and showed no production of any of these metabolites. This indicates that the hepatocytes were responsible for the observed drug metabolism.

[0114] Another drug tested in the hollow fiber liver assist system was acetaminophen, which is metabolized primarily via the Phase II pathway, with production of the glucuronide and sulfate derivatives of acetaminophen by the conjugation enzymes glutathione S-transferase and uridine diphosphate glucuronyltransferase, respectively (see Table 1). A solution of 1 mM acetaminophen/KHB was perfused through the system and the HFMC loaded with Yucatan pig hepatocytes. Samples were analyzed by HPLC, with compounds separated on a Phenyl-Hexyl column (Phenomenex™; length 250 mm x diameter 4.6 mm) and detected at a wavelength of 245 nm using a linear mobile phase gradient. Acetaminophen sulfate and acetaminophen glucuronide first appeared after 1 to 3 hours and increased gradually over 9 hours of sampling to levels of approximately 5 μM and 10 μM, respectively. Again, in an identical control system without hepatocytes, no metabolites were found.

| Table 5. Metabolism of 7-Ethoxycoumarin in the Hollow Fiber Liver Assist System |
|-----------------------------------------------|-----|-----|-----|
| Time (hr) | Concentration (μM) of 7-EC Metabolites |     |     |     |
|    | 7-HC | 7-HCG | 7-HCS |
| 0  | 0.0  | 0.0   | 0.0   |
| 0.6| 0.20 | 0.0   | 0.0   |
| 1  | 0.43 | 0.98  | 0.96  |
| 2  | 0.46 | 1.7   | 1.3   |
| 3  | 0.47 | 2.1   | 1.3   |
| 4  | 0.48 | 2.5   | 1.3   |
| 5  | 0.49 | 2.8   | 1.1   |
| 6  | 0.52 | 3.0   | 1.1   |
| 21 | 0.83 | 3.9   | 0.31  |

[0115] The references cited above are all incorporated by reference herein in their entirety, whether specifically incorporated or not. While this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth as follows in the scope of the appended claims.
CLAIMS

1. A liver assist system that metabolizes one or more compounds in a fluid perfused therethrough, comprising:
   (a) a hollow fiber microfiltration cartridge having (i) an entry port into (ii) an extracapillary space, and an intracapillary space defined by hollow fibers separated from the extracapillary space by membrane walls with selective permeability, which extracapillary space is loaded with viable mammalian hepatocytes, said cartridge coupled to the liver assist system in a cross-flow configuration, so that when the system is operating, fluid enters the extracapillary space via the entry port, contacts said hepatocytes, traverses said membrane walls and exits the cartridge from the intracapillary space via said exit port;
   (b) means for perfusing a fluid through said liver assist system, and
   (c) means for providing oxygen to said hepatocytes.

2. The liver assist system of claim 1 wherein said hepatocytes are aggregated, optionally in the form of spheroids.

3. The liver assist system of claim 1 wherein said mammalian hepatocytes are of porcine, canine, rabbit, murine or primate origin.

4. The liver assist system of claim 1 wherein said mammalian hepatocytes are human hepatocytes.

5. The liver assist system of claim 1 wherein said mammalian hepatocytes are maintained in a viable state for at least twenty four hours.

6. The livers assist system of claim 5 wherein said mammalian hepatocytes are maintained in a viable state for at least four days.

7. The liver assist system of claim 1 which, when operatively connected to a subject with total or partial liver insufficiency is capable of providing substitutive liver function to the subject.

8. The liver assist system of claim 1 perfused with blood, plasma or, serum.

9. The liver assist system of claim 7 operatively connected to said subject.
10. The liver assist system of claim 9 which is connected to said subject by a venous connection.

11. The liver assist system of claim 7 wherein said liver function is characterized as the detoxification of a toxic compound.

12. The liver assist system of claim 11 wherein said compound is selected from the group consisting of ammonia, acetaminophen, lidocaine, phenacetin, chlorzoxazone, 7-ethoxycoumarin, 7-hydroxycoumarin, dextromethorphan, S-mephenytoin, midazolam, testosterone, tolbutamide, and warfarin.

13. The liver assist system of claim 1 wherein said hepatocytes exhibit at least about 10% of the cytochrome P450 enzymatic activity of a similar number of hepatocyte in a healthy liver in vivo.

14. The liver assist system of claim 1 wherein the perfusing means comprises a peristaltic pump coupled to said system.

15. The liver assist system of claim 1 wherein said oxygen providing means comprises a perfusion oxygenator coupled to said system.

16. A process for metabolizing or detoxifying a compound in a fluid, comprising the steps of:
   (a) perfusing said fluid through the liver assist system of claim 1; and
   (b) causing said fluid to contact the viable mammalian hepatocytes in said cartridge under conditions wherein said hepatocytes metabolize or detoxify said one or more compounds.

17. The process of claim 16 wherein said fluid is whole blood, plasma or serum.

18. The process of claim 17 wherein said blood, plasma or serum is from a human.

19. The process of claim 16 wherein said hepatocytes exhibit at least about 10% of the cytochrome P450 enzymatic activity of a similar number of hepatocyte in a healthy liver in vivo.

20. The process of claim 19 wherein said mammalian metabolizing or detoxifying is performed by a cytochrome P450 enzyme in said hepatocytes.
21. The process of claim 16 wherein said compound is selected from the group consisting of ammonia, acetaminophen, lidocaine, phenacetin, chlorzoxazone, 7-ethoxycoumarin, 7-hydroxycoumarin, dextromethorphan, S-mephenytoin, midazolam, testosterone, tolbutamide, and warfarin.

22. The process of claim 16 wherein said hepatocytes are in the form of aggregates, optionally in the form of spheroids.

23. The process of claim 16 wherein said mammalian hepatocytes are of porcine, canine, rabbit, murine or primate origin.

24. The process of claim 16 wherein said hepatocytes are human.

25. The process of claim 16 wherein said hepatocytes are maintained in a viable state for at least about twenty-four hours.

26. The process of claim 25 wherein said hepatocytes are maintained in a viable state for at least about four days.

27. The process of claim 16 wherein a peristaltic pump operatively coupled to said liver assist system actuates said perfusing.

28. A method for metabolizing or detoxifying one or more compounds in a subject, comprising subjecting the blood or plasma of a subject in need of such metabolizing or detoxifying to the process of claim 16 after connecting said subject to the liver assist system.

29. The method of claim 28 wherein said subject suffers from total or partial liver insufficiency.

30. The method of claim 28 wherein connecting is by a venous connection.

31. The method of claim 28 wherein whole blood is subjected to said process.

32. The method of claim 28 wherein said subject is human.
33. The method of claim 28 wherein said one or more compounds is selected from the group consisting of ammonia, acetaminophen, lidocaine, phenacetin, chloroxazone, 7-ethoxycoumarin, 7-hydroxycoumarin, dextromethorphan, S-Mephenytoin, midazolam, testosterone, tolbutamide, and warfarin.

34. The method of claim 28 wherein said hepatocytes are in the form of aggregates, which aggregates are optionally in the form of spheroids.

35. The method of claim 28 wherein a pump operatively coupled to said liver assist system actuates said perfusing.

36. The method of claim 28 wherein said hepatocytes are of murine, rabbit, canine, porcine or primate origin.

37. The method of claim 36 wherein said hepatocytes are of human origin.

38. The liver assist system of claim 1 that also comprises tubing coupled to said entry port, wherein

(a) said viable mammalian hepatocytes are in a culture medium;
(b) said perfusing means is a peristaltic pump suitable for perfusing fluid into said cartridge, through said system, and out of said cartridge; and
(c) said oxygen providing means is a perfusion oxygenator suitable to maintain the viability of said hepatocytes by providing a sufficient concentration of oxygen to the culture medium in which said hepatocytes are incubated.

39. A method of treating blood or plasma to metabolize or detoxify a compound therein using a liver assist system, comprising the steps of:

(a) transporting said blood or plasma into the system of claim 38;
(b) permitting said blood or plasma to contact the hepatocytes in the extracapillary space of said cartridge under conditions wherein the hepatocytes metabolize or detoxify said compound to produce treated blood or plasma,
(c) impelling said treated blood or plasma across said membrane walls into the intracapillary space of said cartridge; and
(d) transporting said treated blood or plasma out of the intracapillary space of said system.
40. A method of providing liver function to a subject in whom liver function is deficient or absent, comprising:
   (a) connecting said subject to the liver assist system of claim 38;
   (b) perfusing said subject's blood or plasma through said system, wherein said hepatocytes metabolize or detoxify compounds which had accumulated in said blood or plasma due to said deficient or absent liver function, to produce treated blood or plasma; and
   (c) returning said treated blood or plasma from said cartridge to said subject.

41. A liver assist system for metabolizing one or more compounds in a fluid perfused therethrough, comprising:
   (a) a rotating bioreactor comprising viable hepatocytes;
   (b) at least one means for delivering oxygen from an oxygenation source connected to said rotating bioreactor to supply oxygen to said viable hepatocytes; and
   (c) means for perfusing said fluid through said liver assist system.

42. The liver assist system of claim 41 wherein said rotating bioreactor is a perfusion rotating wall vessel.

43. The liver assist system of claim 41 wherein said fluid is plasma or serum.

44. The liver assist system of claim 41 wherein said hepatocytes are of porcine, murine, canine, rabbit, or primate origin.

45. The liver assist system of claim 44 wherein said hepatocytes are human hepatocytes.

46. The liver assist system of claim 41 which is capable of providing substitutive liver function to a subject with total or partial liver insufficiency.

47. The liver assist system of claim 46 operatively connected to said subject.

48. The liver assist system of claim 47 wherein said liver assist system is connected to said subject by a venous connection.

49. The liver assist system of claim 41 wherein said hepatocytes exhibit at least about 10% of the cytochrome P450 enzyme activity of healthy intact livers.
50. The liver assist system of claim 41 wherein said hepatocytes exhibit one or more activities of normal hepatocytes \textit{in vivo} selected from the group consisting of plasma protein production, blood coagulation factor production, aromatic amino acid elimination, bilirubin conjugation, glucose metabolism, and heme metabolism.

51. The liver assist system of claim 46 wherein said liver function is characterized as the detoxification of a toxic compound.

52. The liver assist system of claim 51 wherein said one or more compounds is selected from the group consisting of ammonia, acetaminophen, lidocaine, phenacetin, chlorzoxazone, 7-hydroxycoumarin, 7-ethoxycoumarin, dextromethorphan, S-Mephenytoin, midazolam, testosterone, tolbutamide, and warfarin.

53. The liver assist system of claim 41 wherein said hepatocytes are in the form of aggregates, which aggregates are optionally in the form of spheroids.

54. The liver assist system of claim 54 wherein said aggregates have a three-dimensional structure resembling that of organized liver tissue.

55. The liver assist system of claim 41 further comprising at least one hollow fiber microfiltration cartridge connected to said liver assist system in a cross-flow configuration.

56. The liver assist system of claim 41 wherein the perfusing means includes a pump connected to said liver assist system.

57. A method of metabolizing or detoxifying a compound in a fluid, comprising the steps of:
   (a) perfusing said fluid through the liver assist system of claim 41; and
   (b) causing said fluid to contact the viable hepatocytes in said rotating bioreactor under conditions wherein said hepatocytes can metabolize or detoxify said compound, thereby metabolizing or detoxifying said compound.

58. The method of claim 57 wherein said fluid is plasma or serum.

59. The method of claim 58 wherein said plasma or serum is murine, porcine, canine, rabbit, or primate in origin.
60. The method of claim 58 wherein said plasma or serum is human.

61. The method of claim 57 wherein said rotating bioreactor is a perfusion rotating wall vessel.

62. The method of claim 57 wherein said hepatocytes have active cytochrome P450 enzymes.

63. The method of claim 57 wherein said hepatocytes exhibit one or more activities of normal hepatocytes in vivo selected from the group consisting of plasma protein production, blood coagulation factor production, aromatic amino acid elimination, bilirubin conjugation, glucose metabolism, and heme metabolism.

64. The method of claim 57 wherein the compound being metabolized is selected from the group consisting of ammonia, acetaminophen, lidocaine, phenacetin, chlorzoxazone, 7-hydroxycoumarin, 7-ethoxycoumarin, dextromethorphan, S-Mephenytoin, midazolam, testosterone, tolbutamide, and warfarin.

65. The method of claim 57 that further comprises perfusing said fluid through at least one hollow fiber microfiltration cartridge connected to said system in a cross-flow configuration.

66. The method of claim 57 wherein a pump connected to said liver assist system actuates said perfusing.

67. The method of claim 57 wherein said hepatocytes are of mammalian origin.

68. The method of claim 67 wherein said hepatocytes are human.

69. The method of claim 57 wherein said hepatocytes are viable for a period of at least about 24 hours.

70. A method of treating a subject to metabolize or detoxify a compound, comprising connecting a subject in need of such treatment to the liver assist system and carrying out the method of claim 57.

71. The method of claim 70 wherein said connecting is by a venous connection.
72. The method of claim 70 wherein said rotating bioreactor is a perfusion rotating wall vessel.

73. The method of claim 70 wherein said hepatocytes are in the form of aggregates, which aggregates are optionally in the form of spheroids.

74. The method of claim 70 that further comprises perfusing said fluid through at least one hollow fiber microfiltration cartridge connected to said system in a cross-flow configuration.

75. The method of claim 70 wherein a pump operatively connected to said liver assist system actuates said perfusing.

76. The method of claim 70 wherein said hepatocytes are human.