Membrane Bioartificial Organs

Requirements and Experimental Approaches

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In this paper it is reported on the development of membrane bioartificial liver for the treatment of patients with hepatic failure and membrane properties such as selective permeability, hydrophilicity/hydrophobicity and morphology. These are known to play an important role in the definition of biostability, promotion of cell adhesion and functions in order to select and to develop more cytocompatible and biocompatible membranes.

Polymeric semipermeable membranes and membrane processes play a pivotal role in replacement therapy for acute and chronic organ failure and in the management of immunological disease. All clinical extracorporeal blood purification methods employ membrane devices [1]. Membrane processes are effectively used for the intra and extra-corporeal treatment of patients with various pathologies for the removal of endogenous or exogenous toxins from blood (plasmapheresis, hemodialysis; hemo-diafiltration) or for the gas exchange with blood (blood oxygenation) (see Figure 1). The next generation of artificial organs and tissue therapies is almost certain to be similarly grounded in membrane technology.

The adoption of membranes is moreover extremely attractive with systems where biocatalysts in the form of mammalian cells, enzymes or tissue fragments are used. In fact, membranes of suitable molecular weight cut-off are used in bioartificial organs (e.g. pancreas, liver) using isolated cells, as selective barriers to prevent immune system components from getting into contact with the implant, while allowing nutrients and metabolites to permeate freely to and from cells (Figure 1). The use of membranes in bioartificial substitutes and tissue engineering date back to the year 1933, when Vincenzo Bis-

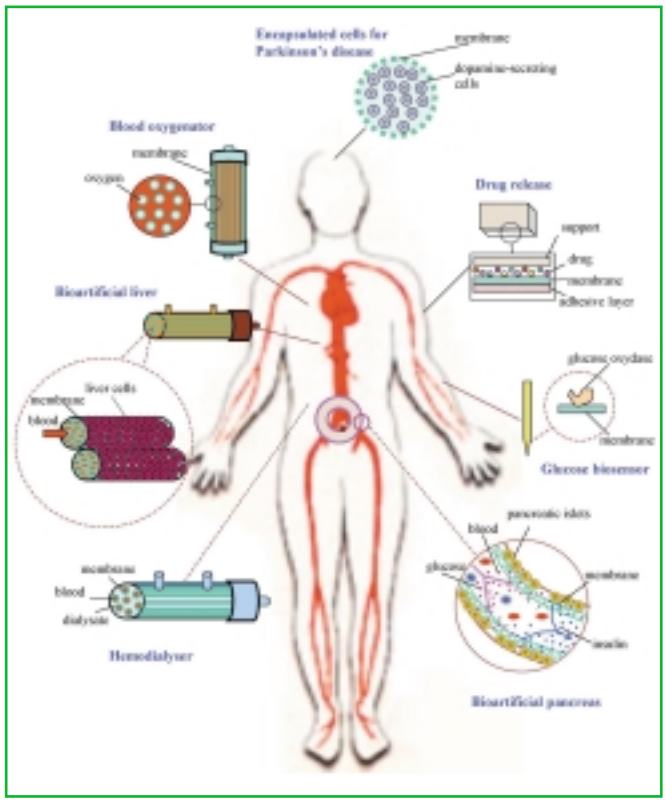

Figure 1 - Membrane biomedical devices

ceglie in Bari, Italy encased mouse tumour cells in a nitro-cellulose membrane and inserted them into the abdominal cavity of guinea pig, to show that cells were not killed by an immune reaction in the pig [2]. Subsequently many researchers focused on the development of immunoprotective membranes to prolong the life of transplant. Currently two important areas of interest are the bioartificial pancreas for the treatment of insulin-dependent diabetes and the liver assist device for the temporary treatment of acute liver failure. Membrane capsules containing dopamine secreting cells also are being explored for treating of Parkinson's disease, a progressive brain disorder characterised by a deficiency of the neurotransmitter dopamine [3]. Immunoprotective membrane cell transplants are being investigated to treat other nervous system disorders. Polymer membranes also are being explored to block cell adhesion or scar tissue formation, for example after surgery, and thus improve wound healing. In addition, the membranes are being investigated for prevention of restenosis (coronary artery narrowing) after angioplasty [2].

In membrane bioartificial organs, cells are compartmentalised

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by means of semipermeable membranes that permit the transport of nutrients and metabolites to cells and the transport of catabolites and specific metabolic products to blood. The membrane must avoid the contact between xenogenic cells and patient's blood to prevent immunological response and rejection of xenograft.
Membranes act as means for cell oxygenation and in the case of anchorage-dependent cells as substrata for cell attachment and culture. In this contribution we report on membrane bioartificial liver, including our experimental experiences. In particular we focused our attention on membrane properties to use in bioartificial organs, on the design of proposed membrane devices as bioartificial liver, and on cytocompatibility of membranes.

Membrane properties

Membranes to use in artificial organs must have mass transfer properties adapted to separation process. Membrane transport properties must permit:
- a controlled removal of solutes and water in the case of hemodialysis and hemofiltration;
- the separation of blood cellular elements from plasma in the plasma separation;
- the addition of oxygen and removal of carbon dioxide in the blood oxygenation.

In bioartificial organs (e.g. pancreas, liver) using isolated cells, membranes provide more important functions, which are essential for the survival of cells. As a result, the type of membrane to use in a bioartificial organ must be chosen on the basis of its permeability characteristics as well as on its physico-chemical properties related to the separation process. In the Table some commercial membrane materials used in artificial and bioartificial organs are reported.
The transport of a chemical specie across membrane is due to a chemical potential difference (difference in temperature, pressure, concentration or combination of all these variables) which acts on system components [4]. In according to the phenomenological approach of irreversible thermodynamics the transport equations of solvent and solute can be expressed as:

$$J_v = Lp(\Delta P - \sigma\Delta\pi) \quad (1)$$

$$J_s = \overline{cs}\,(1-\sigma)J_v + \omega\Delta\pi \quad (2)$$

The transport across a membrane is described from three parameters, hydraulic permeability Lp, solute permeability ω, and the reflection coefficient σ (equations 1 and 2). All these parameters can be experimentally determined. The reflection coefficient is a measure of selectivity of membranes and varies in the range 0-1 for solute freely permeating and completely rejected by membrane respectively. Solute permeability coefficient is related to solute diffusivity in the membrane and membrane thickness. In absence of solute the equation becomes:

$$J_v = Lp\ \Delta P \quad (3)$$

Lp is determined by measuring solvent flux through the membrane under transmembrane hydrostatic pressure differences. Lp is related to the membrane properties as pore size, pore size distribution, pore geometry, thickness, and tortousity. In porous membranes the chemical specie transport depends not only on transmembrane pressure gradient but also on size and shape of the solutes related to the pore size in membrane. The porous membranes used in bioartificial organs are generally microfiltration and ultrafiltration membranes. The pore sizes of microfiltration membranes range from 0.05 to 10 μm. Ultrafiltration is typically used to retain macromolecules from a solution, the lower limit being solutes with molecular weight of few thousand Dalton.
When the hydrostatic pressure difference is zero the transport of a chemical specie through membrane occurs by concentration gradient:

$$J_s = \omega\ \Delta C \quad (4)$$

where

$$\omega = \frac{D_{eff}}{l}$$

As a result, the transport of solutes is obtained as a difference in diffusion rates across the membrane arising from differences in molecular size.
Generally the range of size and physico-chemical properties of solutes which must be transported through membrane in bioartificial organs is extremely wide. Small solutes as electrolytes and oxygen and high MW proteins (70,000 Da) must be efficiently transported through membrane as well as both hydrophilic species dissolved in the plasma and hydrophobic species. As result, the transport of molecules across membranes depends not only on size and physico-chemical properties of molecules related to pore size of membranes but also on solute-membrane interactions.

Type of membranes used in artificial and bioartificial organs

Membrane material	*Therapeutic Indication*
Cellulose and derived (esters, acetates, nitrates)	HD, HDF, HF, BAL
Cuprophan	HD, HDF, BAL
Cellophan	HD, HDF
Ethylvinylalcohol	HD, HDF, BAP
Polyamide	BAL
Polycarbonate	HD, HDF, HF, BAL
Polyimide/poly(ether imide)	BAP
Polymethylmetacrylate	HD, HDF
Polysulfone	HD, HDF, HF, PF BAL
Polyvinilacetate	HD, HDF
Polypropylene	Ox, PF, BAL
Polytetrafluoroethylene	Ox, BAL
Silicone	Ox

HD, hemodialysis; HDF, hemodiafiltration; HF, hemofiltration; Ox, oxygenator; BAP, bioartificial pancreas; BAL, bioartificial liver

Bioartificial liver

Relevance of liver support
Each year in the US, approximately 150,000 people are hospitalised with liver disease and over 43,000 people die from it [5]. These numbers are expected to increase as the 4 million peo-

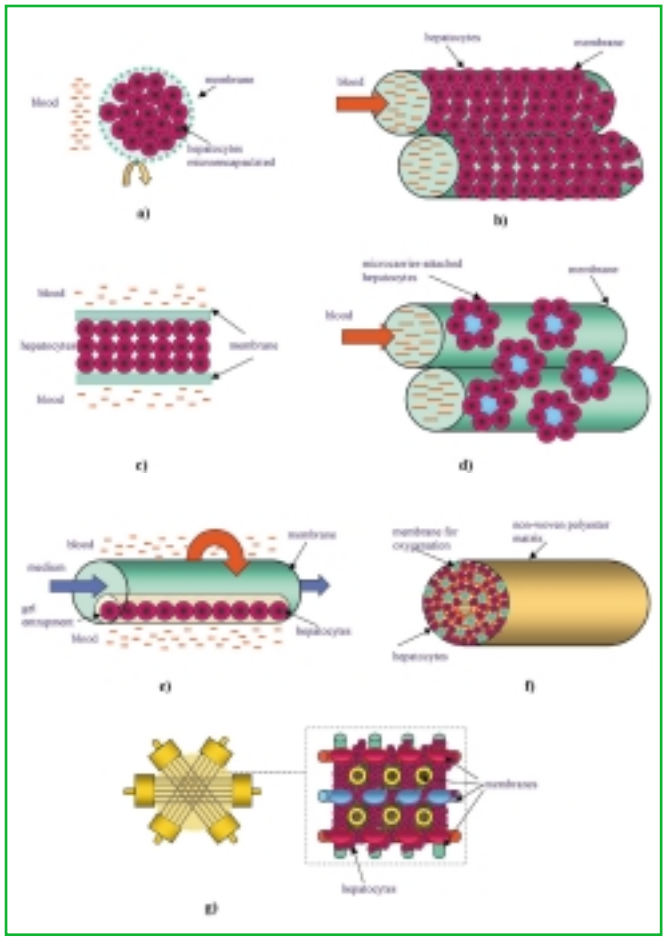

Figure 2 - Schematic of liver support device configurations: a) microencapsulated hepatocytes [10]; b) hepatocytes loaded outside of hollow fibres in extracapillary compartment; c) hepatocytes loaded between flat-sheet membranes [20]; d) microcarrier-attached hepatocytes in the extracapillary compartment [19]; e) hepatocytes loaded in a spirally wound non-woven polyester matrix [16]; f) hepatocytes entrapped in a three-dimensional contracted gel matrix inside of hollow fibres [17]; g) hepatocytes loaded in a network formed by four separate membrane capillaries with different functions [18]

ple currently infected with Hepatitis C advance to liver failure. Transplantation, the only effective means of treating liver failure, is not an option for many patients. Some are simply not sick enough to justify the massive cost, invasiveness and risk of a transplant, leaving them unaided today. Other patients are too sick to qualify, while others die awaiting a transplant.
Ironically, the liver is a highly regenerative organ [6]. Some patients currently undergoing liver transplantation would not need this major surgery if there were a simpler means of obtaining liver function until their own organ had recovered. Over the past 30 years, a variety of supportive therapies for patients with acute liver failure have been proposed. Detoxification based methodologies for liver support as dialysis, hemofiltration, hemoperfusion, have been proven ineffective because physical methods are not sufficient for the management of severe biochemical disorders.
Unlike the other organs (lung, kidney, heart) which have one primary function, the liver has multiple functions essential to maintain life including carbohydrate metabolism, synthesis of proteins, amino acid metabolism, urea synthesis, lipid metabolism, drug biotransformation and waste removal.
To address the critical medical needs of liver-compromised patients, the development of an extracorporeal liver assist device, using isolated liver cells, to which a patient would be temporarily connected until he/she recovered or received a liver transplant could be a promising approach. Components of the patient's blood are to be passed through the device, processed by living liver cells within the device and then returned to the patient using a dialysis-type procedure.
Since fulminant liver failure is potentially reversible, the extracorporeal bridging of liver function would also be beneficial until the patient's own liver resumes functional activity.

Performance of isolated liver cells

Several studies indicate that isolated hepatocytes are capable of supporting all essential hepatic functions and may supply biologically active substances that promote regeneration and repair of the damaged liver being supported. Freshly isolated liver cells the hepatocytes retain many of the metabolic characteristics of the tissue *in vivo*. Thus the introduction of a method of collagenase perfusion introduced by Berry and Friend and modified by Seglen permitted the isolation of hepatocytes from a donor animal liver [7]. The rat liver is not able to provide a sufficient number of cells for a hybrid liver support device, thus the rat liver cell isolation technique was adapted to a hepatocyte isolation on a larger scale. Porcine hepatocytes are one of the best xenograft candidates with regard to differentiated metabolic functions and high-yield retrieval. The development of a primary human hepatocyte cell line is being pursued, however, putative candidates are as yet unavailable for medical use. A potential risk is leakage of tumour cells and their products into patient's circulation.
A very important consideration in liver support system design is the issue of how much liver tissue is needed to provide adequate bioactive support. Data from human studies indicated that the 10% of total liver mass is compatible with life. This value corresponds for a 70 Kg man to approximately to 1.5 $x10^{10}$ hepatocytes [8].
A limiting problem in the development of hybrid liver support device is that hepatocytes lose *in vitro* their metabolic functions with time. As a consequence different technique culture models were introduced. Since anchorage-dependent cells cannot live for long without culture substratum, they must culture in adhesion on some substrata. Appropriate cell adhesion techniques, using artificial substrates as membranes, microcarriers or biological matrix as connective tissue preparations have been studied.

Liver support devices proposed

Two different artificial liver supports are proposed: implantable systems including microcarrier attached hepatocytes, spheroid aggregate hepatocytes, microencapsulated hepatocytes and extracoporeal device including membrane devices [9-12], as is illustrated in Figure 2. The first system is untested in clinical trials as it involves immunosuppressive therapy and the support materials may inhibit reticuloendothelial system.
The first clinical report of bioartificial membrane liver was released in 1987 [13]. This device consisted of a hepatocyte suspension that was separated from the patient's blood by a cellulose acetate dialysis membrane. Since then different extracorporeal device using hollow-fibre membranes or flat-sheet membranes have been proposed. In devices using hollow-fibre mem-

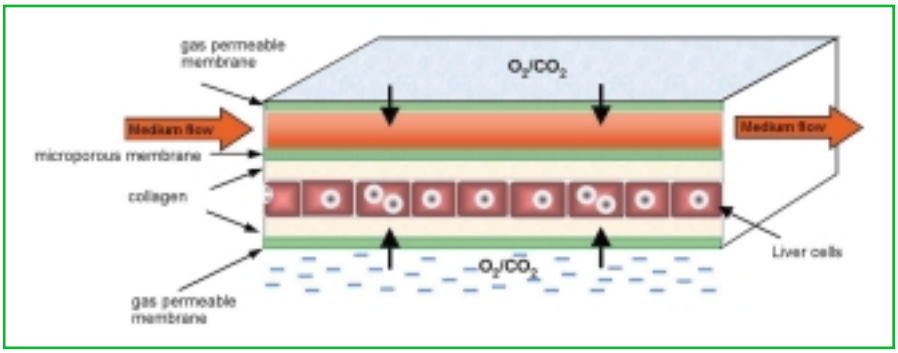

Figure 3 - Schematic of flat membrane bioreactor (FMB) [21-22]

branes, isolated hepatocytes are usually loaded outside of the hollow fibres in the extracapillary compartment, while blood, plasma, or culture medium flow through the lumen of the hollow fibres (Figure 2b). In devices using flat-sheet membranes, hepatocytes are loaded between flat membranes in a sandwich way, while blood or culture medium flow outside of the membranes as shown in Figure 2c. Cells may be free in suspension, attached to walls or attached to microcarrier (Figure 2d).

A key issue concerning the development of bioartificial liver is the maintenance of long-term viability and functions of hepatocytes: oxygen transport resistance and catabolite accumulation may limit the hepatocyte viability and metabolism [7,14]. To overcome the problem of maintenance of viability and liver specific functions *in vitro* of liver cells, Sussmann [15] used immortalised liver cell line in the extracorporeal hollow fibre device as biological element. This device, developed as Hepatix/Baylor unit, consisted of 10,000 individual hollow fibres with cut-off of 70 kDa. This has been tested on 11 patients with fulminant hepatic failure: four patients were sustained until transplant and two survived without the need of transplant and five died for complications. A disadvantage of this unit is the potential of seeding tumour cells into patients. Thus, other researcher proposed new culture models to address the problem of long-term maintenance of liver specific functions of liver cells inside of the bioreactor. Flendrig *et al.* [16], developed a bioreactor based on a spirally wound non-woven polyester matrix, three-dimensional framework for hepatocyte immobilisation and hydrophobic polypropylene membranes for decentralised oxygen supply and CO_2 removal (Figure 2e). Medium or plasma is perfused through the extrafibre space and in direct hepatocyte contact.

Generally many investigators inoculate cells in the extrafibre space. In Nyberg's design hepatocytes are cultured in the hollow fibre lumens [17]. In this device hepatocytes are entrapped in a three-dimensional gel matrix and the extrafibre space of the bioreactor is perfused for 24 hs. with recirculating medium, as is shown in Figure 2f. The blood is passing through the extrafibre compartment and the gel-entrapped cells are perfused by means of medium to improve the supply of oxygen, nutrients, and the removal of catabolites. This device is scaled-up by using hepatocyte spheroids inside polysulfone membranes at University of Minnesota and recently was licensed to Algenix Inc. and it was approved by FDA for Phase I human clinical trials.

Clinical treatment of hepatic failure requires high cell concentration inside of the bioreactor, which is often realised by formation of cell aggregates of large size. In this case resistances to mass transport results in a depletion of oxygen and nutrients in the central cell regions farther away from the external surrounding cell-layers, hence in cell starvation and death. Generally in most of the hollow fibre bioreactors existing to date the transport of nutrients and metabolites is realised by diffusion which is known to be a limiting mechanism of mass transport. In the liver the problem of oxygen and nutrients supply to the cells is solved by arranging them in cell plates with sinusoidal structures located on both sides. An interesting approach was undertaken by Gerlach *et al.* [18] by mixing different fibres in various directions, thus improving the oxygen and nutrients supply to hepatocytes located in between such a network. The Figure 2g shows the scheme of such device [19] licensed to Hybrid Organ GmbH. Four separate capillary membranes with different functions are utilised: plasma inflow by polyamide membrane; plasma outflow by polysulfone membranes; decentralised oxygen supply and carbon dioxide removal with low gradients by polypropylene membranes; sinusoidal endothelial coculture by hydrophilic membrane. This device was used for preliminary clinical studies.

Based on dialysis model, the liver support system developed by Demetriou *et al.* [20] of Cedar Sinai Medical Centre of Los Angeles, consists of a bioartificial liver cartridge that contains billions of pig liver cells and a machine that controls the flow of blood through the cartridge (Figure 3d). The commercial name of the device is "HepatAssist System" of Circe Biomedical and includes cellulose nitrate/cellulose acetate hollow fibre bioreactor containing porcine hepatocytes attached to a collagen-coated microcarriers, two charcoal columns, a membrane oxygenator, and a pump. Plasma from a patient flows through the cartridge. Pig liver cells on the outer surface of the fibres are in contact with the plasma through large pores on the fibre surface. This device was used in USA for a treatment of 6-7 hs. of 25 patients with severe acute liver failure. Of 25 patients, 23 had transplantation within 24 hs. of completing and survived and two died for complications.

These reports encourage the further studies to address the problems related to the development of a bioartificial liver. In fact, although several groups worlwide are creating systems there is not still an ideal device. This is not surprising, given the complexity of liver functions. Liver support system must supply

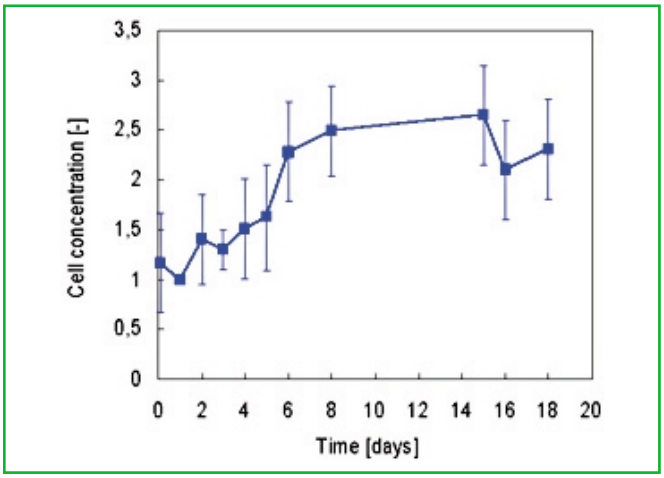

Figure 4 - Time course of pig liver cell concentration in the FMB

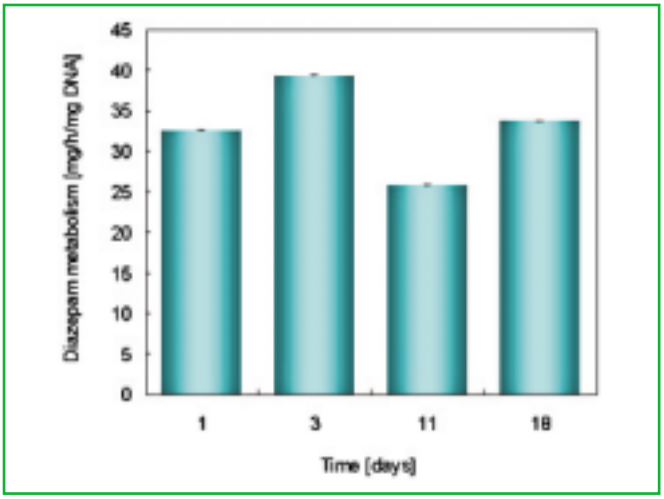

Figure 5 - Rate of diazepam biotransformation of pig liver cells cultured in the FMB in the presence of 10 mg/ml diazepam

various liver specific functions including synthetic and detoxification activities for a time sufficient to allow recovery of a patient or maintenance of patients until transplantation. Some of the current devices do not fully meet these requirements: often they perform well for a limited period of time and rapidly lose their viability and liver specific functions. It is increasingly evident that a multifactorial approach to liver support will be necessary.

Our experience in scale-up membrane bioreactor as bioartificial liver

Recently, we evaluated *in vitro* the performance of a full-scale flat membrane bioreactor (FMB) developed by Bader *et al.,* that permits the culture of liver cells under *in vivo*-like conditions and at high-density culture [21-23]. In such bioreactor porcine hepatocytes are cultured within extracellular matrix between oxygen permeable flat-sheet membranes. Isolated liver cells are located at a distance of 10-20 µm of extracellular matrix. This bioreactor provides culture conditions that improve liver specific functions of liver cells *in vitro.* The FMB is able to provide an *in vivo*-like microenvironment for liver cells: hepatocytes are arranged as a plate in 3-D coculture with intermingled non-parenchymal cells. In contrast to other bioreactors the FMB is based on the organisation of liver cells as a plate within extracellular matrix in which each individual hepatocyte has its own membrane support and thereby its own oxygen supply position (see Figure 3).

In vitro studies demonstrated that the performance of a scale-up FMB using porcine hepatocytes is stable over a period of about 3 weeks and compares well with that of other systems present in literature. Isolated hepatocytes cultured in the FMB reconstitute many of the features of the liver *in vivo*. The cell concentration inside of the FMB increased in the first days of culture and then remained constant until investigated period (Figure 4).

Specific metabolic functions of hepatocytes in terms of albumin synthesis, ammonia elimination and urea synthesis are sustained for the investigated culture time demonstrating thus the long-term maintenance of functional integrity of hepatocytes cultured in the FMB. Consistently the drug biotransformation functions, investigated by using diazepam, were sustained for 18 days of culture at high levels (Figure 5).

Diazepam is metabolised by cytochrome P450 activities, which are among the most sensitive and fragile enzymes found in the hepatocytes, responding quickly by loss of activities to unfavourable culture conditions. The high cell specific activity that found in this study is a clear proof for the workability of the device concept in scale-up. These characteristics encourage to perform *in vivo* experiments in animal models in order to evaluate the potential of the FMB as bioartificial liver.

Cytocompatibility of membranes in bioartificial organs

In membrane bioartificial organs using isolated cells as biological component, semipermeable membranes play more functions: they act as immunoslective barriers, as means for cell oxygenation and provide a large area for cell attachment. All these functions are important for the maintenance of cell viability and specific functions.

In our experimental study we demonstrated that isolated rat liver cells cultured on oxygen-permeable membranes reconstitute many feature of the *in vivo* [24]. As is shown in Figure 6a only cells cultured on polythetrafluoroethylene oxygen-permeable membranes maintained a morphological appearance of hepatocytes similar to their *in vivo* appearance as shown by polyhedric cell shapes and maintenance of cell polarity with distinct bile canaliculi formation.

This condition was maintained in a fully confluent arrangement over the whole period of 14 days. On the contrary, as light microscopic evaluation shows, in cultures on not oxygen-permeable support disintegrating cells were noted and the monolayer is not confluent. The use of gas-permeable membranes permitted the establishment of well defined and prolonged aerobic culture conditions for primary hepatocytes and to reduce ambient PO_2 to 10 % v/v. Such conditions allowed high expression levels of tissue specific functions *in vitro* in terms of albumin secretion, urea synthesis and drug biotransformation functions. This finding is of general relevance for long-term hepatocyte cultures as current practice generally has neglected the aspect of oxygen supply *in vitro* both in batch and in large-scale bioreactor systems.

In a membrane bioartificial organ, cells come into contact with the membrane surface. Therefore, the response of the biological components depends on surface properties of the used membrane. Physico-chemical properties including surface composition, surface charge, surface energy, and surface morphology, may affect cell adhesion and behaviour. Surface properties may affect cell adhesion and metabolism by influencing the ability of the substratum to adsorb proteins and/or by altering the conformation of the adsorbed proteins of the extracellular matrix or by guiding the cell adhesion on the basis of its surface topography.

Recently, studies performed on isolated hepatocytes cultured on semipermeable polymeric membranes have indicated that

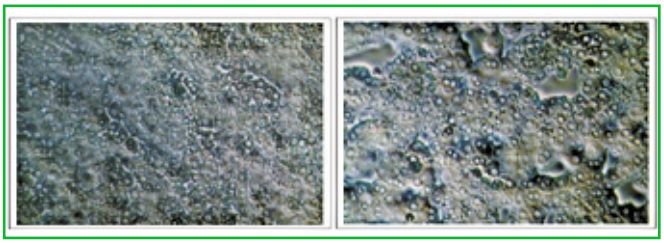

Figure 6 - Inverse light microscopy of rat liver cells cultured on: a) polytethrafluoroethylene oxygen-permeable membrane; b) polystyrene support

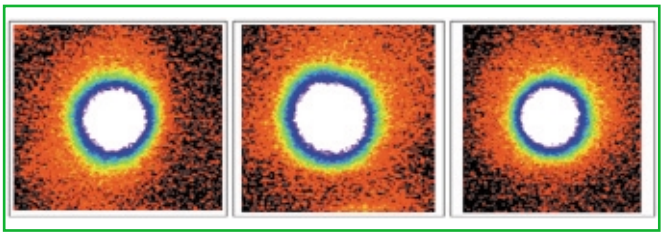

Figure 7 - Fluorescence microscopy of rat liver cells after 3 hs of culture on membranes with different physico-chemical properties: a) polycarbonate membrane; b) perfluoropolymer membrane; c) collagen film

wettable and rougher surfaces enhance adhesion and metabolism of isolated hepatocytes (25-27). The presence of microstructures (i.e. pores) on membrane surface offers attachment points for cell adhesion improving cell viability. Physico-chemical properties play also a dominant role in the modulation of cell-membrane interactions favouring the adsorption and consequent conformational change of important proteins as fibronectin, vitronectin, which are principles mediators of the cell adhesion.

However, different forces are involved in the cell-polymer surface interaction as physical forces and specific receptor-to-surface-protein-ligand interaction. These cellular events are influenced by physicochemical and morphological properties of surface that determine the composition of extracellular matrix proteins. Cells adhere to the surface by interaction of specific receptors, integrins, with adsorbed layer proteins leads to transfer of a signals to the cell interior and may activate genes responsible of protein synthesis and cell proliferation. We investigated adhesion and functions of cells on membranes with different physico-chemical properties. We evaluated the membrane potential of cells by a fluorescent probe with high affinity for DNA (dye propidium iodide). In Figure 7 is shown the fluorescence analysis of cells adhered on perfluoropolymer membrane (PF), polycarbonate (PC) membrane compared with cells adhered on collagen film.

The fluorescent intensity increases when cells are adhered on perfluoropolymer and on polycarbonate membranes with respect to those adhered on collagen, as is reported in the Figure 8. This

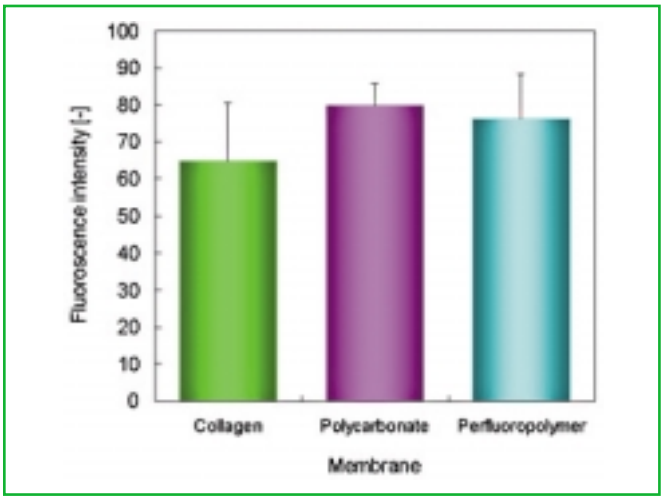

Figure 8 - Fluorescence intensity of rat liver cells after 3 hs of culture on membranes with different physico-chemical properties. Liver cells were incubated in culture medium with 8 mM propidium iodide

means an hyperpolarisation of cytoplasmatic membrane potential due to cell energetic and functional changes occurred during the phases of adhesion and reorganisation of cells on PF and PC membranes. These preliminary results indicated that by changing the type of substrata it is possible to improve the cell adhesion and functions *in vitro*. To this purpose in our laboratory researches on interactions of cells with membranes with different physico-chemical properties are running in order to select and to develop more cytocompatible and biocompatible membranes.

Nomenclature

C = solute concentration, M L^{-3}; $\overline{Cs}$ = mean logarithmic concentration, M L^{-3}; D_{eff} = solute effective diffusion coefficient, L^2T^{-1}; l = membrane thickness, L; Lp = membrane hydraulic permeability, T L^{-1}; P = hydrodynamic pressure, M L^{-1} T^{-2}; Js = solute flux, M L^{-2} T^{-1}; J_v = volume flux, M L^{-2} T^{-1}; π = osmotic pressure, M L^{-1} T^{-2}; σ = Stavermann's reflection coefficient; ω = solute permeability coefficient, LT^{-1}.

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