Section 3 - Publishable results

Publishable summary results:

1. **New HF membranes**

   1.1 Result description:
   ITM-CNR designed and synthesized new membranes in hollow fiber configuration according to the well-known dry-wet spinning method from a polymeric blend of modified polyetheretherketone (PEEK-WC). Investigations in polymeric membrane preparation and in the understanding of their transport properties have made possible the realization of this novel HF membranes for biomedical applications to be used for hepatocytes cultures.

   New PEEK-WC HF membranes yield a useful tool for the reconstruction of liver tissue model *in vitro* and for the reconstruction of liver tissue *in vivo* which allow, on the one side, to understand the mechanisms involved and regulating growth and differentiation, and on the other to study their role in the physiology and pathology of the liver.

   The main advantage and innovation of this exploitable knowledge was the realization by an easy and cheap method of new HF membrane with excellent chemical, thermal and mechanical resistance properties. No market applications yet, the research is at lab prototype level. Collaboration is offered to interested companies for the realization of bioreactor and medical device for liver regeneration.
   Contact: Dr. Loredana De Bartolo, ITM-CNR,Italy;
   e-mail: l.debartolo@itm.cnr.it;loredana.debartolo@cnr.it

2. **Characterisation of the new bioactive membranes**

   2.1 Result description:
   The PEEK-WC membranes in flat and hollow fiber configurations were prepared by optimising the parameters results in order to obtain membranes with specific morphological, physico-chemical and transport properties. Structural characterization of the developed new membranes were performed to manage the design of the biomaterials in order to modulate the own interactions with cells. For this purpose, the morphological property of the novel membranes, such as thickness, porosity and mean pore size were analyzed by scanning electron microscope (SEM), the physico-chemical properties such as the hydrophilicity, wettability and surface free energy were characterized by water contact angle (WCA) and transport properties were investigated in terms of idraulic permeance (J) by measuring the water transmembrane flux (J) vs transmembrane pressure (ΔP^{TM}).

   Among the several types of novel bioactive membranes in flat and hollow fiber configuration, only those with morphological, physico-chemical and transport properties, which allow interactions with hepatocytes and ensure a good and selective transfer of metabolites, were chosen as eligible and good candidate for biomedical applications.

   The main advantage and innovation of this exploitable knowledge was the characterisation as a useful tool and the driving power for the design and realisation of new bioactive membranes.

   No market applications yet, the research is at lab prototype level. Collaboration is offered to interested companies for the realization of bioreactor and medical device for liver regeneration.
   Contact: Dr. L. De Bartolo, ITM-CNR, Italy,
   e-mail: l.debartolo@itm.cnr.it;loredana.debartolo@cnr.it

3. **Plasma process optimization and immobilisation procedures** (PLASMA DEPOSITION METHOD OF A BIODEGRADABLE FUNCTIONAL COATING (D5))
   It is a class of coating that can be plasma deposited by using the lactide conventional monomer used for polymerization of polilactic acid. By tuning plasma parameters (thus chemical structure and thickness of the coating) it is possible to tune the degradation time in water media. Further research is necessary, with specific target in mind, to adapt this coating to bioreactor applications, or the prosthesis, or to drug delivery. No market applications yet, the research is at lab prototype level. Collaboration is offered to interested companies.

Contact Prof. Pietro Favia, Department of Chemistry, University of Bari, favia@chimica.uniba.it

4. Bioreactor prototype
(and 8. Design and realisation of the bioreactor using membrane and biodegradable scaffold)

4.1 Result description:

In order to evaluate the performance of the developed bioactive polymeric materials different bioreactor prototype will be developed as dynamic tissue culture environment in which tissue specific mechanical forces (pressure, shear stress, interstitial flow), homogeneous and constant conditions will be applied and in which human hepatocytes were cultured in a optimal microenvironment allowing the simulation of the *in vivo* hepatic organization.

Morphological and functional behaviours of human hepatocytes were investigated in a gas permeable membrane bioreactor and in a small scale bioreactor. In this both bio-hybrid system cells were cultured on the novel bioactive membranes which were located in tightly contact with a gas-permeable membrane foil, that ensures the optimal transfer of CO_2, O_2 and H_2O vapour to the cells adhered on the membranes and to the medium overlaying the cells. In this operating conditions human hepatocytes were cultured in a optimal microenvironment allowing the simulation of the *in vivo* sinusoidal organization displaying an enhanced metabolic activity for the entire culture time and an evidenced cell reorganization strongly similar to that observed in a *in vivo* liver tissue.

Morphological and functional behaviours of human hepatocytes were investigated furthermore in a new crossed HF membrane bioreactor developed by ITM-CNR and based on the use of PEEK-WC and polyethersulfone (PES) HF membranes with different molecular weight (MW) cut-off and physico-chemical properties and cross assembled in alternating perpendicular manner. Hepatocytes were cultured in the extraluminal compartment among the PEEK-WC HFs, devoted to provide the cells oxygenated medium containing nutrients and metabolites, and the PES HFs devoted to remove from cell compartment catabolites and cell specific products. In this way the two HF membrane systems mimic the *in vivo* arterious and venous blood vessels. The morphological behaviour of the human hepatocytes after 18 days of culture in the bioreactor evidenced an high level of cell adhesion with a dense layer of cells on the fibers. The establishment of cell-cell contacts was observed and hepatocytes appeared to be mostly polygonal in shape and surrounded by an extracellular matrix-like structure. An enhanced metabolic activity was maintained at high levels over the entire culture time demonstrating the good performance of the bioreactor to maintain viability and functional integrity of human hepatocytes.

The main advantage and innovation of this exploitable knowledge was the realization of bioreactor prototype as device with a controlled environment that may provide an inexpensive and reliable *in vitro* physiological model for studying engineered liver tissue constructs. All the membrane bioreactor prototype tested represent an important advantageous tool to obtain useful information about the morphology and functions of *in vitro* reconstructed tissue at molecular levels, providing an *in vivo* very similar microenvironment able not only to elicit specific cellular responses but to also to study disease, infection and therapeutic effects of drugs and proinflammatory cytokines.

No market applications yet, the research is at lab prototype level. Collaboration is offered to interested companies for the realization of *in vitro* models for drug testing and medical device for liver regeneration.

Contact Prof. Pietro Favia, Department of Chemistry, University of Bari, favia@chimica.uniba.it

5. Surface coatings with functional groups and immobilisation of biomolecules (PLASMA-FUNCTIONALIZATION OF SURFACES FOLLOWED BY IMMOBILIZATION OF PEPTIDES (D6))

It is a class of deposition and/or treatment plasma processes that allow to provide biomedical surfaces with proper functional groups to be utilized for the covalent (direct or through a spacer molecule) or non covalent (e.g., through self-asembly) immobilization of RGD and other peptides. By tuning plasma parameters (thus the distribution of chemical groups and the thickness of the modified layer) and the variables (pH, temperature, time, etc) of the immobilization reaction it is possible to tune the density of the biomolecules immobilized at the surface of the material. In certain cases a network of peptide is

obtained, mimicking the extracellular matrix to some extent. No market applications yet, the research is at lab prototype level. Collaboration is offered to interested companies for bioreactors, biosensors and other applications where the controlled immobilization of a peptide is needed.

Contact Prof. Pietro Favia, Department of Chemistry, University of Bari, favia@chimica.uniba.it .

6. Design and preparation of tailor-made self-assembling peptides (IQS)

During this project IQS designed and synthesized three new self-assembling peptide scaffolds containing peptide motifs for proper hepatocyte instruction. The material was highly soluble in water and produced solutions at pH 3.5 when dissolved at a concentration of 1% (w/v). After buffering the solutions at pH 7.0 with PBS they turned into gels. The peptides were used to study their nanostructure characteristics by atomic force microscopy (AFM). In all cases, the modified peptides formed fibers as observed by AFM and were capable of forming hydrogels. The three new peptide sequences designed and synthesized are depicted in Table 5.

	Peptide name	Peptide sequence	Nanofibre	Gel
1	**YIG**	AcN-**YIGSR**-GG-(RADA)$_4$-CONH$_2$	Yes	Yes
2	**RGD**	AcN-**GRGDSP**-GG-(RADA)$_4$-CONH$_2$	Yes	Yes
3	**TAG**	AcN-**TAG**SCLRKFSTM-GG-(RADA)$_4$-CONH$_2$	Yes	Yes

Table 5: Sequences of the self-assembling peptides which were designed and synthesized within WP 5.

The ability of peptides to support the maintenance of liver-specific function by culturing hepatocytes in a peptide sandwich fashion similar to the broadly standardized collagen sandwich culture was tested within the WPs 8 and 9 (see below).

Contact: Dr. Carlos E. Semino, IQS; E-mail: semino@MIT.EDU

7. Characterization of the developed bioactive scaffold materials (IQS)

IQS studied the physicochemical properties of the peptide scaffold RAD16-I with respect to its functional counterparts (see Table above). In particular, the concentration-temperature dependence of it structure stability was investigated. It was observed that the peptide suffer structural transition from β-sheet to random coil after thermal treatments. Moreover, higher concentrations of peptide were "protected" to the thermal treatment maintaining their main β-sheet structure, suggesting that the β-sheet to random coil transition is evidenced only at low concentration because the peptides are displaced from the self-assembling peptide fiber into the solution and subsequently change to the random coil configuration. This finding indicates that in certain conditions the peptides can change their secondary structure and therefore become sensitive to the enzymatic treatment since random coil structures are easy to be degraded by proteases. Low concentrations of peptide solutions were treated with trypsin following the analysis of their degradation products by high performance liquid chromatography-electrospray ionization mass spectrometry (HPLC-MS-ESI), and the expected products after RAD16-I treatment were obtained after trypsin treatment. Finally, the peptides, once characterized, there were used to develop layers to culture fresh rat hepatocytes and maintain them in sandwich format.

Contact: Dr. Carlos E. Semino, IQS; E-mail: semino@MIT.EDU

8. Design and realisation of the bioreactor using membrane and biodegradable scaffold (UL)

A bioreactor with a gas-permeable membrane (PTFE) at the bottom has been constructed which guaranteed the maintenance of liver-specific functions in primary hepatocyte cultures This novel device was shown to be well suited for studying a variety of drugs (Schmitmeier et al., 2006). This small-scale bioreactor with an oxygen-permeable PTFE-membrane has been suitable for simulating the hyperoxic situation with the aim of *in vitro* screening of new compounds for potential antioxidant capacity (Schmitmeier et al.,(2007).

UL and ITM-CNR have established small-scale bioreactors connected to a perfusion system which was tested using primary animal and human hepatocytes cultured on the oxygen-permeable PTFE- and PEEK-

WC-PU membranes under a dynamic condition. Primary human hepatocytes cultured on the PEEK-WC-PU membrane were shown to perform liver-specific functions for prolonged periods.

No market applications yet, the research is at lab prototype level. Collaboration is offered to interested companies.

Contact: Prof. Dr. Augustinus Bader, BBZ, University of Leipzig, Email: augustinus.bader@bbz.uni-leipzig.de

9. **Set-up of the perfusion system** and

10. **Device for monitoring and control of the bioreactor(UL)**

Before to use all the designed membrane bioreactors as devices for the long-term maintenance of human hepatocytes culture, tracer experiments without the cells were performed in order to investigate, characterize and optimize the bioreactor fluid dynamic properties. The bioreactors were connected to a perfusion system consisting of a glass medium reservoir, tubing, a micro-peristaltic pump and a glass medium waste. The tracer, consisting in a solution of William's culture medium, was sent to the bioreactor with specific and constant flow rate. The bioreactors were challenged by changing the tracer concentration stepwise in the feed stream (C_{in}) and the outlet concentration (C_{out}) was continuously monitored by on-line spectrophotometer. The fluid dynamics of the bioreactors were characterized in terms of the cumulative residence time distribution (RTD) to step inputs.

The main advantage and innovation of the set-up of the perfusion systems was the fluid dynamic modelling and characterisation as useful tools which permit to culture liver cells under controlled fluid dynamics conditions and which allow an easier and more reliable estimation of the metabolic rate unaffected by mass transport. Therefore, the metabolite concentration at the cell compartment would equal that in the outlet stream and could be determined by assaying the effluent.

No market applications yet, the research is at lab prototype level. Collaboration is offered to interested companies for the realization of bioreactor and medical device for liver regeneration.

Contact: Prof. Dr. Augustinus Bader, UL, Email: augustinus.bader@bbz.uni-leipzig.de

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11. Standardisation of isolation and freezing protocols of human liver cells (UL)

Due to the limited availability of human livers UL established first a protocol for freezing primary animal hepatocytes immobilized in the mini bioreactor. Comparison of the parameters for functional levels of cryopreserved and unfrozen (control) hepatocytes revealed that the cryopreservation process has no major impact on the expression of liver-specific functions with respect to protein synthesis, detoxification and biotransformation. A protocol for the cryopreservation of primary animal hepatocytes, as well as primary human hepatocytes (provided by Admet Technologies) immobilized in the small-scale bioreactor has been established providing the maintenance of hepatocyte functionality following cell thawing.

The main advantage and innovation of this exploitable knowledge was regarding the freezing protocol since full assembly bioreactor could be frozen.

No market applications yet, the research is at lab prototype level. Collaboration is offered to interested companies.

Contact: Prof. Dr. Augustinus Bader, BBZ, University of Leipzig, Email: augustinus.bader@bbz.uni-leipzig.de

12. **Evaluation of viability and specific functions of cell liver in culture on the bioactivepolymeric membranes and in scaffold into bioreactor (UL, ITM-CNR)**

In order to evaluate the performance of the developed bioactive polymeric materials the viability and specific functions of primary human hepatocytes were evaluated culturing cells on native and modified membranes, in flat configuration as well as on HF membranes, in the different developed bioreactor prototypes. The morphological behaviours of cells were evaluated by scanning electron microscopy (SEM) and by laser scanning confocal microscopy (LSCM). The maintenance of liver specific functions was investigated in terms of urea and albumin synthesis. Drug biotransformation was assessed using Diazepam as model drug.

A high level of cell adhesion with a dense layer of cells on the different biomaterials tested was evaluated. Hepatocytes appeared to be mostly polygonal in shape and surrounded by an extracellular matrix-like structure. The reorganization of cytoskeleton proteins like actin and vinculin in focal adhesion, the morphological behaviuor of the cells with a polyhedral shape, the presence of lumen like structures surrounded by the hepatic bile duct marker CK19 and the secretion of ECM proteins such as laminin and fibronectin by human hepatocytes cultured on membranes evidenced a cell reorganization strongly similar to that observed in a *in vivo* liver tissue. Human hepatocytes cultured in all the developed bioreactors displayed an enhanced metabolic activity for the entire culture time and for a long period time. The metabolic pathway of diazepam biotransformation including the formation of its metabolites temazepam, oxazepam and nordiazepam occurred and all these metabolites were generated in all the membrane bioreactors tested. In particular oxazepam was produced to a larger extent with respect to other metabolites confirming the ability of the bioreators to sustain a high conversion of intermediates nordiazepam and temazepam to the final metabolite, as occur in humans. The sustained diazepam biotransformation was furthermore a clear evidence that human hepatocytes expressed at high levels the individual CYP isoforms involved in the drug biotransformation. These enzymes are among the most sensitive and fragile found in hepatocytes, responding quickly by loss of activities to unfavourable culture conditions.

The main advantage and innovation of this exploitable knowledge was the workability of the device concept and the good performance of the novel bioactive membrane and of all the bioreactor prototypes to maintain viability and functional integrity of human hepatocytes.

High viability and metabolic function of primary rat hepatocytes and rat embryonic liver cells cultured on native and NH3-grafted membrane bioreactors was also confirmed.

No market applications yet, the research is at lab prototype level. Collaboration is offered to interested companies for the realization of bioreactor and medical device for the long-term maintenance of human liver cells as an *in vitro* models for drug testing and as medical device.

Contact… Prof. Dr. Augustinus Bader, BBZ, University of Leipzig, Email: augustinus.bader@bbz.uni-leipzig.de

13. Establishment of genome and proteome data bank for hepatocytes in culture (UL-IQS)

Genome of rat primary hepatocytes was evaluated by microarrays and RT-PCR measurements.

Total RNA was isolated from cultured primary rat hepatocytes using the RNeasy Mini Kit (Qiagen). Arrays have been hybridized and scanned by IZKF Leipzig, Core Unit DNA-Technologies. We have used liver cells from three rats each analyzing cell suspension at day 0 (control), and cells cultured and harvested at day 3 on native and NH3-modified membrane, as well as on collagen sandwich, thus, resulting in overall 12 arrays (n=3, four conditions). The ArrayAssist 5.5.1 (Stratagene) software was used to analyze row data. There were no big differences in gene expression of rat hepatocytes cultivated in these three systems. Very interestingly, 59 among 31000 genes are significantly changed in rat hepatocytes cultured on all three different supports: 23 genes were up-regulated while 36 genes down-regulated. So, NH3-plasma modification of the PEEK-WC-PU membrane had no effect on the gene expression. RT-PCRs measurements confirmed results of microarrays. This is the first study on genomics of hepatocytes cultivated in bioreactors.

Protein samples were prepared from rat embryonic (E-17) liver (RLC-18) cells for proteomic analyses by 2D-gel electrophoresis and mass spectrometry. 2DE-analyses were performed in WITA Teltow, Berlin. Then the samples and sent to Dr. Lange and Mr. Schulze for further analyses at the University of Erlangen.

Protein samples isolated from rat embryonic liver cells were tested for the differences in the expression of proteins for embryonal development , proliferation and differentiation, liver specific functions (albumin, urea, glutathione S transferase), oxidative stress,, inflammation (cytokines), antioxidant system (reduced glutathione, glutathione peroxidase, and thioredoxin reductase), acute-phase-response enzymes, etc. The differences in protein expression between samples cultivated in convential plates and PEEK-WC-PU membrane bioreactors were observed. Our method was considered to be suitable for a concomitant 2-DE analysis in *in vitro* toxicity studies. This is first work on proteome of rat liver embryonic cells. We also conduceted first study on proteome of rat liver (in vivo).

However, more studies should still be undertaken to evaluate and compare the complete secreted proteome and expression patterns of total liver-specific genes in hepatocytes cultured on the PEEK-WC-PU membranes and in collagen and peptide scaffolds.

No market applications yet, the research is at lab prototype level. Collaboration is offered to interested companies.
Contact: Prof. Dr. Augustinus Bader, BBZ, University of Leipzig, Email: augustinus.bader@bbz.uni-leipzig.de

14. Design of microarrays/mass spectrometry for better specifity (UL-IQS)

Microarray analysis was conducted during 3. year of the project at the microarray core facility of the Interdisziplinäres Zentrum für klinische Forschung (IZKF) Leipzig (Faculty of Medicine, University of Leipzig).. 5 µg of total RNA were used to prepare double-stranded cDNA (Superscript II, Life Technologies, Gaithersburg, MD USA) primed with oligo-dT containing an T7 RNA polymerase promoter site (Genset SA, Paris, France). cDNA was purified by phenol-chloroform extraction before in vitro transcription using the IVT labeling kit (Affymetrix, Santa Clara, CA, USA) to synthesize cRNA. After the in vitro transcription, unincorporated nucleotides were removed using the RNeasy kit (QIAGEN, Hilden, Germany). The cRNA was fragmented and hybridized to Affymetrix GeneChip HG133A array. The washing and staining of the probe array was performed according to the manufacturer's instructions. The array was scanned with a third generation affymetrix GeneChipScanner. Affymetrix GeneChip data have represented about 31,000 transcripts from approximately 28,700 well-substantiated rat genes with complete Rat Genome coverage were extracted from fluorescence intensities and were scaled in order to normalize data for inter-array comparison using MAS 5.0 software according to the instruction of the manufacturer (Affymetrix).

For proteomic analyses, protein was isolated from cultured RLC-18 cells using peq Gold TriFast[TM] Reagent (peqlab, Erlangen, Germany) according to the manufacturer`s protocol. Proteins were analyzed by two-dimensional electrophoresis and mass spectrometry. Proteomics analyses are performed in WITA Teltow, Berlin. Then the samples and sent to Dr. Lange and Mr. Schultze for further analyses at the University of Erlangen.
The protein-pellet were suspended in 80 µl AP-buffer (9 M Urea), 2 µl were used for a BCA-assay. The whole sample were processed and dried in a Speedvac. Following DTT and Servalyte were added.
The running conditions for the IEF: 1 h at 100 V; 1 h at 200 V; 17.5 h at 400 V; 1 h at 600 V; 30 min at 1000 V; 10 min at 1500 V; 5 min at 2000 V; voltage (mA) and power rating (W) were limited to 300. IEF-start with 0.7 mA; ended with 1.2 mA. The 2nd dimension running conditions are 15 min 65 mA; 6.5 h 130 mA (2 gels were handled in one run). Staining: silver (Ag Blum) Gels were scanned with 300 dpi. For evaluation of the gel-images the software "Melanie 7" from the Swiss Institute of Bioinformatics in Switzerland was used.
The proteins, contained in the polyacrylamide-gels as spots, were picked from the gels (spot picking) and destained by kit. We used the ProteoSilver[TM] Plus Silver Stain Kit from Sigma. After destaining the gel – pieces were tryptic digested overnight. After digesting the proteins into peptides they were analyzed by mass spectrometry with the AXIMA-CFR[PLUS] from Shimadzu.
It uses a nitrogen-laser with 337 nm wave length, pulse-modulation 3 ns, max. pulse-rate 10 Hz. Ion detachment take place in a 1.2 m flight tube or a 2.25 m flight tube (reflectron). Ion detection by SEV. The sensitivity has been defined for 50 fmol experimentally.
Calibrations were performed by external standards. For proteomics PMF and/or PSD mass spectra's were used. The peptides were put with dried droplet method on a target. As matrix we used alpha-cyano-4-hydroxy-cinnamic-acid. Pulseextraction was set to m/z 2300.
The PMF's were taken in m/z range from 1000 to 3500, but in effect masses greater than 2500 Data are often to diffuse and show bad resolution. So we mostly use the mass range from 1000 to 2500 for protein identification. The spectra's were identified with the help of the Mascot – databank, we used here software from Mascot, named"Mascot Distiller". For PSD-spectra's we used a m/z range of 100 to 3500. The collision – gas used was nitrogen.
The main advantage and innovation of this exploitable knowledge was that promising future proteome research can be based on these results.
No market applications yet, the research is at lab prototype level. Collaboration is offered to interested companies.
Contact: Prof. Dr. Augustinus Bader, BBZ, University of Leipzig, Email: augustinus.bader@bbz.uni-leipzig.de

15. Profile of toxic response of cells in the bioreactor to a range of toxins (UL-IQS)

Rat embryonic liver cells, grown on PEEK-WC-PU-membrane bioreactors have been shown to more resistant to paracetamol and coumarin hepatotoxicity in comparison with conventional collagen plates. A proteomic study was also conducted showing differential susceptibility of RLC-18 cells cultivated in bioreactors toward coumarin toxicity giving rise to utility of PEEK-WC-PU-membrane biorecators in liver tissue engineering for hepatotoxicity evaluations.

No market applications yet, the research is at lab prototype level. Collaboration is offered to interested companies.

Contact: Prof. Dr. Augustinus Bader, BBZ, University of Leipzig, Email: augustinus.bader@bbz.uni-leipzig.de