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Tesi

Realizzazione di membrane polimeriche innovative per la crescita *in vitro* di tessuti umani

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Sommario

L'enorme progresso delle conoscenze nel campo della biologia cellulare e delle biotecnologie ha consentito, negli ultimi anni, lo sviluppo di tecnologie mirate alla coltivazione ed alla ricostruzione *in vitro* di tessuti od organi, definendo una nuova branca di scienze biomediche conosciuta con il termine di "ingegneria dei tessuti". Questa tecnologia permette di poter far crescere cellule autologhe *ex vivo* e riutilizzarle nella riparazione di lesioni e rigenerazione di tessuti mediante coltura in matrici polimeriche biocompatibili tridimensionali. Modulando opportunamente le caratteristiche chimiche, meccaniche e fisiche di tali matrici è possibile teoricamente rigenerare *in vitro* tipi diversi di tessuti. Queste strutture bioartificiali rappresentano la seconda generazione di sistemi di sostituzione di organi e tessuti. La prima generazione era essenzialmente costituita da organi artificiali tradizionali (reni, macchina cuore-polmoni, protesi valvolari cardiache, pacemakers cardiaci protesi di articolazione ileo-femorale e ginocchio), la cui alternativa clinica è il trapianto di organi umani ottenuti da donatori. L'ingegneria tessutale rappresenta un'evoluzione di tali interventi terapeutici consentendo la possibilità di associare la potenzialità del trapianto di cellule viventi con la tecnologia degli organi artificiali per la realizzazione di strutture funzionali. Tale strategia implica lo studio sia delle strutture dei costrutti e delle forze fisiche che su questi agiscono, sia dei fattori biochimici e molecolari della crescita e del differenziamento delle cellule e dei tessuti. I prodotti dell'ingegneria dei tessuti derivano direttamente da queste ricerche ed hanno lo scopo di condurre a dispositivi che associano le strutture artificiali con quelle viventi, rendendone disponibili le importanti funzioni.

Lo studio dei materiali utilizzati nell'ingegneria tessutale rappresenta un importante settore di ricerca. I materiali naturali hanno spesso il vantaggio di contenere nella

loro struttura, in particolari *sequenze segnale*, informazioni atte a promuovere l'adesione delle cellule e a mantenerne le funzioni. D'altra parte i materiali di sintesi, nonostante permettano la riproducibilità delle procedure di produzione ed un preciso controllo di alcuni parametri (ad esempio, peso molecolare, tempi di degradazione, idrofilicità o idrofobicità della superficie di contatto), possono talvolta interagire in maniera indesiderata con le cellule. Quando i materiali di sintesi si fondono con i materiali naturali, si realizzano materiali dotati di elevate caratteristiche e prestazioni denominati biomateriali innovativi.

Tra le diverse aree di ricerca che concorrono a formare le competenze in questo campo un ruolo fondamentale, è quello svolto dalla biologia cellulare. E' infatti, sempre più necessario comprendere nei dettagli i meccanismi che regolano la crescita e la differenziazione cellulare e le modalità attraverso le quali i componenti della matrice extracellulare interagiscono con le funzioni cellulari. La cellula è, infatti, l'unità strutturale e funzionale comune a tutti gli organismi viventi e ne possiede tutte le caratteristiche. L'organismo umano non fa eccezioni ed è costituito da miliardi di cellule organizzate tra loro per formare tessuti, organi ed apparati.

Grande importanza in questa organizzazione la rivestono le membrane cellulari che, oltre a compartimentare l'interno della cellula e a rivestire i diversi organuli, ne regolano le complesse interazioni con l'ambiente esterno. Le membrane sono strutture dinamiche e complesse che regolano, in modo estremamente selettivo, il traffico di molecole dall'esterno verso, l'interno della cellula e viceversa. La permeabilità altamente selettiva nei confronti di soluti polari consente di regolare e mantenere una determinata concentrazione ionica all'interno della cellula. Le membrane non sono strutture statiche ed inerti; al contrario, esse rappresentano sistemi complessi nei quali molecole di tipo diverso si associano tra loro costituendo una struttura ordinata. Il ruolo complesso delle membrane cellulari diventa particolarmente evidente negli organismi pluricellulari, per i quali i rapporti ed i contatti non si limitano esclusivamente all'ambiente esterno, come avviene

nell'organismo unicellulare, ma interessano anche le altre cellule che compongono i tessuti ed i diversi organi. Grazie alle membrane, le cellule possono comunicare tra loro e scambiarsi messaggi che consentono di armonizzare le attività da svolgere in modo correlato. Le comunicazioni tra cellule possono avvenire in modo diretto oppure per mezzo di impulsi elettrici o di messaggeri chimici altamente specifici che generano risposte altrettanto specifiche, legate alle caratteristiche della membrana. Il messaggio ormonale, ad esempio, consiste nella secrezione, da parte di una ghiandola opportuna, di particolari messaggeri chimici che, immessi, nel flusso sanguigno, sono recepiti in modo altamente specifico e selettivo da particolari strutture denominate *recettori*, presenti sulla membrana delle cellule bersaglio. L'interazione tra ormone e recettore provoca una modificazione strutturale delle proteine di membrana con conseguenti modificazioni dell'attività metabolica cellulare. In particolare, è importante sottolineare come la membrana costituisca la struttura di riconoscimento e di contatto tra cellule e biomateriali.

Per la grande importanza che rivestono le membrane cellulari la scelta d'elezione nel campo dei biomateriali innovativi, sono le membrane polimeriche che per definizione costituiscono una barriera selettiva con la capacità di garantire scambi controllati di nutrienti, di ossigeno e di prodotti di scarto tra il microambiente cellulare e l'esterno.

Lo scopo di questa tesi è la neomorfogenesi di un tessuto. Perché la neomorfogenesi *in vitro* abbia successo, è necessario creare un ambiente favorevole nel quale le cellule siano in grado non solo di riprodursi, ma anche di entrare in contatto ed organizzarsi tra loro in modo da formare il nuovo tessuto. Questo lavoro di tesi si è concentrato sulla possibilità di rigenerare il tessuto nervoso ed epatico. I materiali usati sono polimeri biodegradabili. I polimeri biodegradabili possono essere naturali o sintetici. Il criterio generale di selezione dei polimeri per le applicazioni biomediche è di unire le proprietà meccaniche ed il tempo di degenerazione alle necessità del tipo di impiego. Il polimero ideale deve: 1) avere proprietà meccaniche

che siano compatibili con il tipo di applicazione, restando inalterato per il tempo necessario al tessuto circostante per ripararsi; 2) non evocare una risposta infiammatoria o tossica; 3) essere metabolizzato dal corpo non appena ha raggiunto il suo scopo, senza lasciare traccia; 4) essere facilmente maneggevole per il raggiungimento del prodotto finale; 5) essere facilmente sterilizzabile.

I materiali utilizzati sono stati il chitosano, un amino polisaccaride (poli 1,4 D-glicosamino), un derivato parzialmente deacetilato della chitina, il polimero strutturale primario nell'esoscheletro degli artropodi; il policaprolattone, un polimero sintetico biodegradabile e il poliuretano, largamente conosciuto ed utilizzato nel campo biomedicale.

L'innovazione di questa tesi è stata quella di utilizzare tecniche ampiamente utilizzate come l'inversione di fase, per la creazione di membrane microstrutturate e altamente specializzate per la crescita e lo sviluppo di tessuti *in vitro*.

Summary

Restoration and replacement of damaged tissue have greatly progressed and contributed significantly to surgery in the twentieth century. Especially, tissue reconstruction is still one field of important research, since the goal of producing perfect artificial tissue has not been achieved. The difficulties encountered in repairing or replacing severely damaged tissue may be resolved through a process called tissue engineering. Tissue engineering is a rapidly emerging field that combines the established disciplines of engineering, biology, and medicine with the goal of fabricating biological substitutes that restore, maintain, or improve tissue function. It has the potential to produce a bioartificial organ and tissue substitutes that can grow with the patient. This should lead to a permanent solution to the damaged organ or tissue without the need for supplementary therapies, thus making it a cost-effective treatment in the long term. Although initially targeted for applications in regenerative medicine, a novel application of this technology has been to generate experimental model systems for studying biological mechanisms and testing the efficacy of potential therapies. In particular, this very promising technique involves the in vitro seeding and attachment of human cells onto a material. These cells then proliferate, migrate, and differentiate into the specific tissue while secreting the extracellular matrix (ECM) components required to create the tissue. It is evident, therefore, that the choice of material is crucial to enable the cells to behave in the required manner to produce specific tissues and organs. Different materials have been proposed to support cells and promote their differentiation and proliferation toward the formation of a new tissue.

The design and selection of a biomaterial is a critical step in the development of scaffolds for tissue engineering. Generally, the ideal biomaterial should be nontoxic, biocompatible, promoting favorable cellular interactions and tissue development, while possessing adequate mechanical and physical properties. In addition, it should

be biodegradable and bioresorbable to support the reconstruction of a new tissue without inflammation.

During the 1960s and the 1970s, a first generation of materials was developed for use inside the human body. These early biomaterials must have been used to achieve a suitable combination of physical properties that match those of the replaced tissue with a minimal toxic response in the host. In 1980, there were more than 50 implanted devices in clinical use made from 40 different materials. A common feature of most of the materials was their biological inertness.

By the mid-1980s bioactive materials which had begun to be clinically used in a variety of orthopedic and dental applications were developed. Another advance in these bioactive materials was the development of resorbable biomaterials that exhibited clinically relevant controlled chemical breakdown and resorption. Improvements in these bioinert, bioactive, and resorbable biomaterials are limited because all biomaterials used for repair or restoration of the body represent a compromise – living tissue can respond to changing physiological loads or biochemical stimuli, but synthetic materials cannot. This limits the lifetime of artificial body parts. Recently, the next third-generation biomaterials were designed to stimulate specific cellular response at the molecular level. The separate concepts of bioactive materials and resorbable materials were converged. Molecular modification of resorbable polymer systems elicits specific interactions with cell integrins and thereby direct cell proliferation, differentiation, and ECM production and organization.

Polymeric materials have greatly contributed to the development of bioactive and biodegradable materials. Progress in both membrane and cell culture technology has also greatly contributed to the success of artificially engineered tissue. It was demonstrated that polymeric membranes are attractive for their characteristics of selectivity, stability, and biocompatibility in the use of biohybrid systems for cell culture. In particular, semipermeable membranes act as supports for the adhesion of

anchorage-dependent cells and allow the specific transport of metabolites and nutrients to cells and the removal of catabolites and specific products.

In tissue-engineered constructs, the surface and transport properties of the membranes play an important role in the promotion of cell adhesion, proliferation, and viability. The material surface properties, such as chemical composition, hydrophilicity/ hydrophobicity, charge, free energy, and roughness, affect cell adhesion through the modulation of proteins secreted by cells or contained in the physio- logical liquids.

Recently, the advantages of both natural and synthetic polymers have been combined in strategies whereby critical amino acid sequences from natural polymers are grafted onto syntethic polymers. Polymeric membranes processing is another key issue. Many implants are made of composite materials or highly organized structures; methods of manufacturing such implants reproducibly may be crucial to their success.

The aim of this work is to make a new generation of polymeric membranes with precisely engineered surfaces and properties will be used to provide the desired biological response *in vivo*

or to act as biodegradable scaffold for tissue transplantation, by well known technique as phase inversion.

Chapter I

Tissue engineering

1.1 Introduction

The loss or failure of an organ or tissue is a frequent, devastating, and costly problem in health care, occurring in millions of patients every year. Organ or tissue loss is currently treated by transplanting organs from one individual to another or performing surgical recostruction by transferring tissue from one location in the human body to the diseased site. Although these therapies have saved and improved millions of lives, they remain imperfect solutions.

Tissue engineering represents a new, emerging interdisciplinary field applying a set of tools at the interface of the biomedical an engineering sciences that use living cells or attract endogenous cells to aid tissue formation or regeneration [1] to restore, maintain, or improve tissue function.

Engineered tissues using the patient's own (autologous) cells or immunologically inactive allogeneic or xenogeneic cells offer the potential to overcome the current problems of replacing lost tissue function and to provide new therapeutic options for diseases such as metabolic deficiencies.

1.2. Structure and function of normal tissue.

Biological tissue is composed of three basic components : cells, intercellular substances, especially extracellular matrix, and various body fluids. In all tissue, cells are assembled during embryonic development into coherent groupings by virtue of specific cell-cell and cell-matrix interactions. Each type of tissue has a distinctive pattern of structural organization adapted to its particular function, which is strongly influenced by metabolic [2] and /or mechanical factors [3;4].

1.2.1 Basic Tissues

Humans have more than 100 distincly different types of cells variously allocated to four types of basic tissues. (Table 1).

Basic Tissues	Examples
Epithelial tissue	
Surface	*Skin epidermis, gut mucosa*
Glandular	*Thyroid follicles, pancreatic acini*
Special	*Retinal or olfattory epithelium*
Connective tissue	
Connective tissue proper	*Skin dermis*
Loose	*Pericardium, tendon*
Dense (regular, irregular)	*Adipose tissue*
Special	*Bone marrow, blood cells*
Hemopoietic tissue, blood and lymph	
Supportive tissue	*Cartilage, bone*
Muscle tissue	
Smooth	*Arterial or gut smooth muscle*
Skeletal	*Limb muscolature, diaphram*
Cardiac muscle	*Heart*
Nerve tissue	*Brain cells, peripheral nerve*

TABLE 1: THE BASIC TISSUES: CLASSIFICATION AND EXAMPLES.

(FROM *BIOMATERIALS SCIENCE: AN INTRODUCTION TO MATERIALS IN MEDICINE, 2ND EDITION 2004*)

The basic tissues play specific functional roles and have distinctive microscopic appearances.

They have their origins in embryological development; early events include the formation of a tube with a three layers in its wall: (1) an outer layer of ectoderm, (2)

an inner layers of endoderm, and (3) a middle layer of mesoderm (Fig.1).

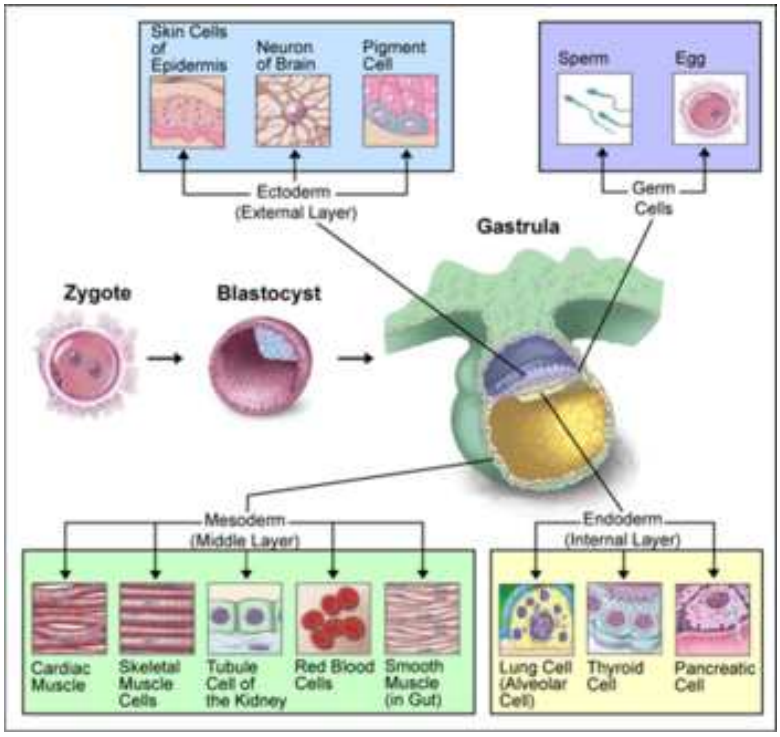

FIGURE1: EARLY PHASE OF EMBRYOLOGICAL DEVELOPMENT (HTTP://CREATIONWIKI.ORG/PRENATAL_DEVELOPMENT).

Epithelium covers the internal and external body surfaces. It provides a protective barrier (e.g., skin epidermis) on an absorptive surface (e.g., gut lining), and can generate internal and external secretions (e.g., endocrine and sweat glands, respectively). Epithelium derives mostly from ectoderm and endoderm, but also from mesoderm.

Epithelia accommodate diverse functions. An epithelial surface can be (1) a protective dry, cutaneous membrane; (2) a moist, mucose membrane, lubricated by glandular secretions; (3) a moist, membrane lined by mesothelium, lubricated by fluid that derives from blood plasma; and (4) the inner lining of the circulatory system, called endothelium. Epithelial cells play fundamental roles in the directional movement of ions, water, and macromolecules between biological compartments, including absorption, secretion, and exchange.

Supporting the other tissues of the body, connective tissue arises from mesenchyme, a derivative of mesoderm. Connective tissue also serves as a scaffold for the nerves and blood vessels that support the various epithelial tissue. Other types of tissue with varying functions are also mesenchymal origin. These include dense connective tissue, adipose tissue, cartilage and bone,and circulating cells, as well as inflammatory cells that defend the body against infections organism and other foreign agents.

Muscle cells develop from mesoderm and are highly specialized for contraction. They have the contractile proteins actin and myosin in varying amounts and configurations, depending on cell function. Muscle cells are of three types: smooth muscle, skeletal muscle, and cardiac muscle. The latter two have a striated microscopic appearance, owing to their discrete bundles of actin and myosin organized into sarcomers. Smooth muscle cells, which have a less compact arrangement of myofilaments, are prevalent in the walls of blood vessels and the gastrointestinal tract. Their slow, nonvoluntary contraction regulates blood vessel calibre and proper movement of food and solid waste, respectively.

Nerve tissue, which derives from ectoderm, is highly specialized with respect to irritability and conduction. Nerve cells not only have cell membranes that generate electrical signals called action potentials, but also secrete neurotransmitters, molecules that trigger adjacent nerve or muscle cells to either transmit an impulse or to contract.

1.2.2. Extracellular Matrix

Extracellular matrix (ECM) comprises the biological materials produced by, residing between, and supporting cells. ECM, cells, and capillaries are physically integrated in functional tissue. The ECM holds cells together by providing physical support and a matrix to which cells can adhere, signal each other, and interact. ECM consists of large molecules synthesized by cells,exported to the intercellular space and linked together into a structurally supportive composite. ECM is composed of (1) fibers

(collagen and elastin) and (2) a largely amorphous interfibrillary matrix (mainly proteoglycans, noncollagenous cell-binding adhesive glycoproteins, solutes, and water). The principal functions of the ECM are:

Mechanical support for cell anchorage

Determination of cell orientation

Control of cell growth

Maintenance of cell differentiation

Scaffolding for orderly tissue renewal

Establishment of tissue microenvironment

Sequestration, storage, and presentation of soluble regulatory molecules.

ECM consists of large molecules interlinked to form a reticulum that schematically resembles a fiber- reinforced composite; in reality, ECM forms an expansible glyco-protein-water gel held in dynamic equilibrium by fibrillar proteins. The key constituents of ECM include fibrillar proteins such as collagen and elastin, amorphous matrix components exemplified by glycosaminoglycans (GAGs) and proteoglycans, and adhesive proteins such as fibronectin and laminin.

Collagen comprised a family of closed related but genetically, biochemically, and functionally distinct molecules, which are responsible for tissue tensile strength. The most common protein in the animal world, collagen provides the extracellular framework for all multicellular organism.

Amorphous intercellular substances contain carbohydrate bound to protein. The carbohydrate is in the form of longchained polysaccharides called glycosaminoglycans (GAGs). When GAGs are covalently bound to proteins, the molecules are called proteoglycans. GAGs are highly charged (usually sulphated) polysaccharide chain up to 200 sugars long, composed of repeating unbranched disaccharide units (one of which is always an amino sugar- hence the name

glycosaminoglycan). GAGs are divided into four major groups on the basis of their sugar residues:

Hyaluronic acid: a component of loose connective tissue and of joint fluid, where it acts as a lubrificant

Chondroitin sulfate and dermatan sulfate

Heparan sulfate and heparin

Keratin sulfate

Adhesive proteins, including fibronectin, laminin, and entactin permit the attachment and movement of cells within the ECM.

Fibronectin is a ubiquitous, multidomain glycoprotein possessing binding sites for a wide variety of other ECM components, including collagen, heparins A and B, fibrin, and chondroitin sulfate. Fibronectin's adhesive character also makes it a crucial component of blood clotting and of pathways followed by migrating cells. Thus, fibronectin rich pathways guide and promote the migration of many kinds of cells during embryonic development and wound healing.

Laminin is a n extremely abundant component of the basal lamina, a tough, thin, sheet like substratum on which cells sit. This protein is important for cell differentiation and tissue remodelling [5].

1.3 Tissue engineering as an approach to replace lost tissue or organ function

In the most frequent paradigm of tissue engineering, isolated living cells are used to develop biological substitutes for the restoration or replacement of tissue or organ function. Generally, cells are seeded on bioadsorbable scaffolds, a tissue is matured *in vitro*, and the construct is implanted in the appropriate anatomic location as a prothesis. Cells used in tissue engineering may come from a variety of sources including application-specific differentiated cells from the patients themselves (autologous), human donors (allogeneic) or animal sources (xenogeneic), or undifferentiated cells comprising progenitor or stem cells. The use of isolated cells or

cell aggregates allows the manipulation prior to implantion, e.g., transfection of genetic material or modulation of the cell surface in order to prevent immunorecognition. Three general strategies have been adopted for the creation of new tissue including cell injection, closed or flow-throught systems, and tissue engineering using biodegradable scaffolds.

1.3.1 Cell injection Method

The cell injection method avoids the complications of surgery by allowing the replacement of only those cells that supply the needed function. Isolated, dissociated cells are injected into the bloodstream or a specific organ of the recipient.

The transplanted cells will use the vascular supply and the stroma provided by the host tissue as a matrix for attachment and reorganization [6]. This method offers opportunities for a number of applications in replacing metabolic functions as occurs in liver disease, for example [7]. However, cell mass sufficient to replace lost metabolic functions is difficult to achieve and its application for replacing functions of structural tissue such as heart valves or cartilage is rather limited. Several cells types may be used for injection, such as bone marrow cells, blood-derived progenitor cells, and muscle satellite cells.

Whole bone marrow contains multipotent mesenchymal stem cells (marrow stromal cells) that are derived from mesoderm and are involved in the self maintenance and repair of various mesenchymal tissue. These cells can be induced *in vitro* and *in vivo* to differentiate into cells of mesenchymal lineage, including fat, cartilage and bone, and cardiac and skeletal muscle. The first successful allogenic bone marrow transplant in a human was carried out in 1968. More than 40,000 transplant (from bone-marrow, peripheral blood, or umbilical cord blood) were carried out worldwide in 2000 (www.ibmtr.org/newsletter/pdf/2002Feb.pdf).

1.3.2 Closed-system method

Closed system can be either implanted or used as extracorporeal devices. In this approach, cells are isolated from the body by a semipermeable membrane that allows

diffusion of nutrients and the secreted cell products but prevents large entities such as antibodies, complement factors, or other immunocompetent cells from destroying the isolated cells. Protection is also provided to the recipient when potentially pathological (e.g. tumorigenic) cells are transplanted. Implantable systems (encapsulation systems) come in a variety of configurations, basically consisting of a matrix that protects the cells and supports their survival and function through a surrounding porous membrane (Fig. 2).

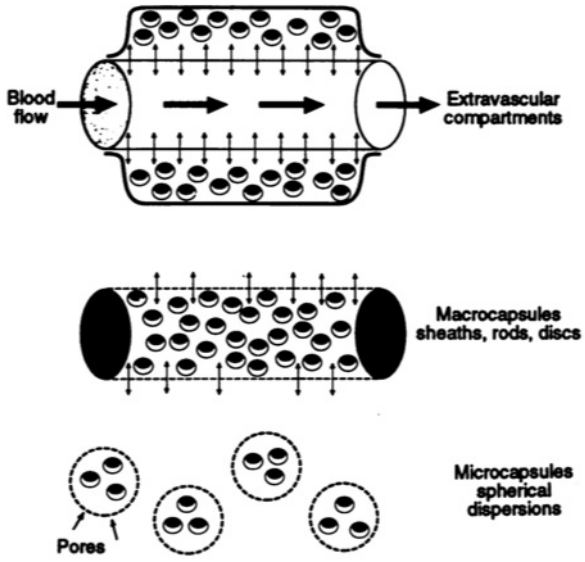

FIG.2. THERE ARE THREE COMMON CLOSED-SYSTEM CONFIGURATIONS FOR CELL TRANSPLANT DEVICE [8].

In vascular –type designs the transplanted secretory cells are housed in a chamber around a vascular conduit separated from the bloodstream by a semipermeable membrane. As blood flows through, it can absorb substances secreted by the therapeutic cells while the blood provides oxygen and nutrients to the cells. In macroencapsulation systems, a semipermeable membrane is used to encapsulate a relatively large (up to 50-100 million per unit) number of transplanted cells. Microcapsules are far more durable than microcapsule droplets and can be designed to be refillable in the body. Moreover, they can be retrieved, providing opportunities for more control than microcapsules.

Their main limitation is the number of cells they can accommodate. In animal experiments, implantable closed-system configurations have been successfully used for the treatment of Parkinson's disease as well as diabetes mellitus [9-11]. Major drawbacks of these systems are fibrous tissue overgrowth and resultant impaired diffusion of metabolic products, nutrients and wastes, as well as the induction of a foreign-body reaction with macrophage activation resulting in destruction of the transplanted cells within the capsule [12].

In extracorporeal systems (vascular or flow-through designs) cells are usually separated from the bloodstream. Great progress in being made in the development of extracorporeal liver assist devices for support of patients with acute liver failure.

1.3.3. Tissue engineering using biomaterial scaffolds.

Open systems of cells transplantation with cells being direct contact to the host organism aim to provide a permanent solution to the replacement of living tissue. The rationale behind the use of open systems is based on empirical observations: dissociated cells tend to reform their original structures when is given the appropriate environmental conditions in cell culture. For example, capillary endothelial cells from tubular structures and mammary epithelial cells form acini that secrete milk on the proper substrata *in vitro* [13]. Although isolated cells have the capacity to reform their respective tissue structure, they can do that wuth a limited degree since they have no intrinsic tissue organization and are hindered by the lack of a template for guiding the reconstruction. Moreover, tissue cannot be transplanted in large volumes because diffusion limitations restrict interaction with the host environment for nutrients, gas exchange, and elimination of waste products. Therefore, the implanted cells will survive poorly more than a few hundred microns from the nearest capillary or other source of nourishment [14]. With these observations in mind, an approach has been developed to regenerate tissue by attaching isolated cells to biomaterials that serve as a guiding structures for initial tissue development. Ideally, these scaffold materials are biocompatible, biodegradable into nontoxic products, and manufacturable [15]. Natural materials used in this context are usually composed of

extracellular matrix components (e.g., collagen, fibrin) or complete decellularized matrices. Synthetic polymer materials are advantageous in that their chemistry and material properties (biodegradable profile, microstructure) can be well controlled. In general, these concepts involve harvesting of the appropriate cell types and expanding them *in vitro*, followed by seeding and culturing them on the polymer matrices.

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Chapter II

Polymeric membranes as biomaterials

2.1 Introduction

For the new therapeutic strategy, it is indispensable to provide cells with a local environment that enhances and regulates their proliferation and differentiation for cell-based tissue regeneration.

Biomaterials plays an important role in the creation of this cell environment[1].

At the dawn of 21st century, biomaterials are widely used throughout medicine, dentistry and biotechnology. Just 50 years ago biomaterials as we think of them today did not exist. The word "biomaterials" was not used.

A definition of "biomaterial" endorsed by a consensus of expert in the field, is :

> *A biomaterial is a nonviable material used in a medical device, intended to interact with biological system.[2]*

If the word "nonviable" is removed, the definition becomes even more general and can address many new tissue-engineering and hybrid artificial organ applications where living cells are used.

Indeed, a complementary definition essential for understanding the goal (i.e., specific and applications) of biomaterials science is that of "biocompatibily".

> *Biocompatibility is the ability of a material to perform with an appropriate host response in a specific application[2].*

This general concept of biocompatibility has been extended recently in the approach of "tissue engineering" in which *in vitro* and *in vivo* pathopysiological processes are harnessed by careful selection of cells, materials, and metabolic and biomechanical conditions to regenerate functional tissues.

The design and selection of a biomaterial is a critical step in the development of scaffolds for tissue engineering. Generally, the ideal biomaterial should be nontoxic, biocompatible, promoting favorable cellular interactions and tissue development, while possessing adequate mechanical and physical properties. In addition, it should be biodegradable and bioresorbable to support the reconstruction of a new tissue without inflammation [3].

Biomaterials can be divided into four major classes of materials: polymers, metals, ceramic

(including carbons, glass-ceramics, and glasses), and natural materials (including from both plants and animals). Sometimes two different classes of materials are combined together into a composite material. Such composites are a fifth class of biomaterials.

New materials have been designed *de novo* specifically for medical use, such as biodegradable polymers and bioactive ceramics.

The word “biodegradation” is defined to be the phenomenon where a material is degraded or water solubilised by any process in the body to disappear from the site implanted. There are two ways of materials disappearance. First, the main chain of the material is hydrolysed or enzymatically digested to decrease the molecular weight, and finally disappears. Second, the material is chemically cross-linked to form a hydrogel insoluble in water. When the cross-linking bond is degraded to generate water-soluble fragments, the fragments are washed out from the site implanted, resulting in the disappearance of material. Synthetic polymers are generally degraded by simple hydrolysis while natural polymers are mainly degraded enzymatically.

Some are derived from existing materials fabricated with new technologies, such as polyester fibers that are knit or woven in the form of tubes for use as vascular grafts, or cellulose acetate plastic that is processed as bundles of hollow fibers for use in artificial kidney dialysers. Some materials are “borrowed” from unexpected sources

such as pyrolytic carbons or titanium alloys that had been developed for use in air and space technology. And other materials are modified to provide special biological properties, such as immobilization of heparin for anti-coagulant surfaces.

The field of biomaterials is in the midst of a revolutionary change in which the life sciences are becoming equal in importance to materials science and engineering as the foundation of the field. Simultaneously, advances in engineering (for example nanotechnology) are greatly increasing the sophistication with which biomaterials are designed and have allowed fabrication of materials with increasingly complex functions. Such sophisticated materials are often designed to mimic a subset of the physicochemical properties of natural materials. Increasingly, nature inspires not only the materials themselves but also the means by which they are made. Inspiration for the design of new biomaterials has been derived from structure–function analysis on various length scales of the extracellular materials that cells use to organize themselves into tissues [4].

2.2 Polymers

Polymers represent the largest and most promising class of biomaterials. This is attested by their widespread use in various medical applications. A large number of polymeric biomaterials have been developed and new developments continue to appear in both open and patent literatures [5].

Many types of polymers are widely used in biomedical devices that include orthopedic, dental, soft tissue, and cardiovascular implants.

The key of success of polymer-based biomaterials is the relative easy of preparation coupled with low cost with which they can be synthesized with wide range of properties and functionality [6].

The choice of a material for a specific application is mainly based on its physicochemical, interfacial and biomimetic properties, although traditional mechanical properties such as impact strength, elasticity and permeability are also essential for specific applications [7].

Indeed, the choice of the polymer is primarily governed by the end use of the biomaterial and involves selection not only on the basis of physical and chemical properties but also on extensive biochemical characterization followed by specific preclinical testing of the chosen material [8,9].

Polymer may be derived from natural sources, or from synthetic organic processes. Naturally derived polymers are abundant and usually biodegradable. Their principal disadvantage lies the development of reproducible production methods, because their structural complexity often makes modification and purification difficult. Additionally, significant batch-to-batch variations occur because of their "bio preparation" in living organism (plants, crustaceans).

Synthetic polymers are available in a wide variety of compositions with readily adjusted properties. Processing, copolymerization and blending provide simultaneous means for optimizing a polymer's mechanical characteristics and its diffusive and biological properties (Table 2).

Both natural and synthetic polymers are long-chain molecules that consist of a large number of a small repeating units. Polymers can be either amorphous or semycristalline. They can never be completely crystalline owing to lattice defects that form disordered, amorphous regions. The tendency of a polymer to crystallize is enhanced by the small side groups and chain regularity. The presence of crystallites in the polymer usually leads to enhanced mechanical properties, unique thermal behaviour, and increased fatigue strength.

These properties make semicrystalline polymers (often referred to simply as crystalline polymers) desiderable materials for biomedical applications.

The state of polymer is very important relative to its mechanical, chemical, thermal and permeation properties. In the liquid or melt state, a non-crystalline polymer possesses enough thermal energy for long segments of each polymer to move randomly (Brownian motion). As the melt is cooled, a temperature is eventually reached at which all long-range segmental motion cease. This is the glass transition temperature (T_g), and it varies from polymer to polymer. Polymers used below their T_g tend to be hard and glassy, while polymers used above their T_g are rubbery. Polymers with any crystallinity will also exhibit a melting temperature (T_m) owing to melting of the crystalline phase.

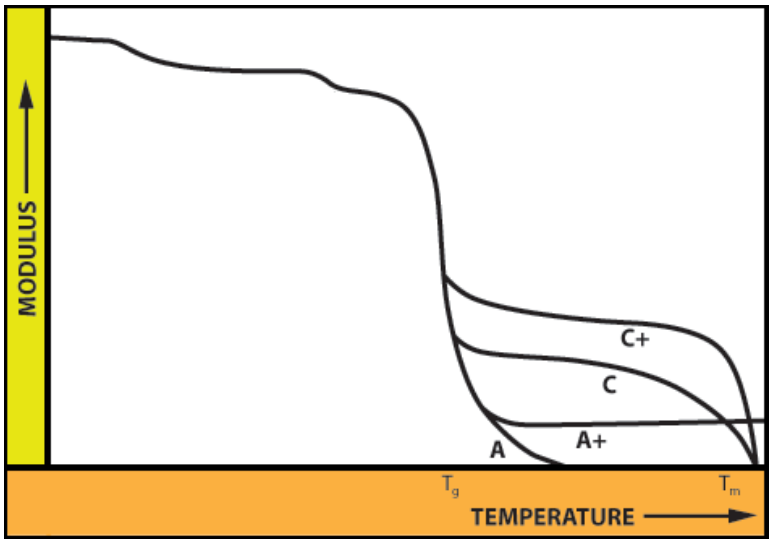

FIG.3 TENSILE MODULUS AS A FUNCTION OF THE TEMPERATURE FOR GENERIC POLYMER. A = AMORPHOUS POLYMER; A+ = AMORPHOUS POLYMER, HIGH MOLECULAR WEIGHT; C = CRYSTALLINE POLYMER; C+ = CRYSTALLINE POLYMER, HIGHER DEGREE OF CRYSTALLINITY.

Two regions can be distinguished in figure 3 of a completely amorphous polymer: the glassy state with a high modulus and the rubbery state with a modulus, which is often three to four orders of magnitude lower. The mobility of the polymeric chains is very restricted in the glassy state, since the segments cannot rotate freely around the main chain bonds. On increasing the temperature, some motions can occur in the side chains or in a few segments of the main chain. However, these are only marginal changes with the density of the polymer decreasing to a limited extend (or conversely the specific volume increasing a little). At the glass transition temperature the thermal energy is just sufficient to overcome the interactions between the chains. For this

reason, important parameters which determine the position of the glass transition are chain flexibility and chain interaction. In the rubbery state the segments can rotate freely along the main chain bonds, implying a high degree of rubbery state is discontinuous. In addition to the glass temperature, another important parameter, the degree of crystallinity, also determines the state of the polymer. The influence of crystallinity on the tensile modulus is depicted in figure 3, curves C and C+. In the glassy state the mechanical properties are little influenced by presence of crystallites. On passing through the glass transition temperature the amorphous glassy state is transformed into rubbery state but the crystalline phase remains unchanged, i.e. the chains remain in the crystal lattice which maintains its rigidity until the melting temperature has been reached. Hence, for a perfect crystalline polymer (100% crystallinity) changes in the modulus are most likely at the melting temperature (T_m) rather than the glass transition temperature (T_g). A large number of polymers are semi-crystalline. In such polymers the glassy phase exhibits the same mechanical properties as for a completely amorphous polymer. However, in the rubbery state the mechanical properties will depend on the crystalline content of the polymer. Generally the modulus of a semi-crystalline polymer decreases as a function of temperature (curve C). This figure also depicts the modulus of a completely crystalline polymer (curve C+) indicating that no rubbery state is observed in this case and that the modulus only decreases drastically at the melting point [6,10].

TABLE 2:

Materials frequently used in soft tissue engineering applications

Origin	Polymer (family)
Natural	Collagen (component of the extra cellular matrix-ECM) Fibrin Gelatin Poly(hydroxybutyrate) Polysaccharides (most common are hyaluronic acid, chitosan, starch and alginates)
Synthetic	Poly(esters) (most common are poly(α-hydroxy acids): poly(lactic acid) (PLA) and poly(glycolic acid) (PGA)) Poly(ε-caprolactones) Poly(propylene fumarates) Poly(anhydrides) Poly(orthoesters)

2.3 Polymeric Membrane

Basically, all polymers can be used for preparing membranes but the chemical and physical properties differ so much that only a limited number will be used in practice.

Membrane technology is of major importance in medical applications, in particular in a number of life saving treatment methods. Membranes are used in drug delivery, artificial organs, tissue regeneration, diagnostic devices, as coatings for medical devices, bioseparations, etc. Only in the US for example, the medical membrane market approaches 1.5 billion dollars per years and grows steadily. The biggest part

of the medical market involves membranes in drug delivery, hemodialysis, other artificial organs (oxygenators, pancreas, etc.) and tissue engineering.

Generally, biomaterial-based membranes that are in contact with biological fluids should prevent any type of infection and immune response, blood clotting and other biological responses that could affect the properties of the fluid and, therefore, the patient. For this reason, it is important to know both *host* and *material response* for a certain biomaterial. The host response is usually related to inflammation, fibrosis, coagulation and hemodialysis. The material response focuses on fracture, wear, corrosion, dissolution, swelling and leaching.

A wide range of natural and synthetic materials is used in biomedical membrane applications. Biocompatible polymers can be divided into several categories, based upon changes in host response [11]: (i) inert biomaterials that exhibit little or no host response; (ii) interactive biomaterials that are designed to trigger specific and beneficial responses such as cell growth, adhesion; (iii) viable materials that at implantation, for instance, incorporate or attract living cells that are considered as normal tissues by the host and are actively resorbed by the system; and (iv) replant biomaterials that consist of *in vitro* cultured tissue from the patient's cells [12].

Erosion (degradability) is also a key parameter for materials that are used as implants and/or in tissue regeneration. Swelling and leaching result from diffusion. Swelling involves transport of ions or fluid from the tissue into the biomaterial. As a consequence of swelling, the elastic limit of a material can be reduced leading to static fatigue or crazing [11]. Leaching takes place if, for instance, one component of the biomaterial dissolves into the surrounding fluid phase. This can cause local biological reactions to the released products, reduced fracture strength and elastic modulus of the material. The dissolution varies depending on the nature of the polymer (hydrophilic/hydrophobic). Hydrophobic polymers, for example, dissolve preferably in the amorphous regions, which results in increased area, integrity loss and release of small particles. Bioresorbable polymers are designed to degrade within

the body and be adsorbed naturally when its function has been accomplished [13]. These degradation characteristics differ from polymer to polymer, and can vary from swelling to dissolution by hydrolysis, for instance, when being exposed to body fluids. Bioresorbable materials degrade products that are normal metabolites of the body. Some examples of degradable polymers are polylactide, polyglycolide, polycaprolactone and polyhyaluronic acid esters, but also natural polymers like collagen, chitosan.

2.3.1. Fabrication methods.

A great variety of well-known membrane fabrication techniques are used in tissue engineering applications. Several fabrication methods based on polymer casting are frequently applied to produce tissue engineering membrane, e.g. liquid induced phase separation (LIPS, immersion precipitation)[14-16], thermally induced phase separation (TIPS)[17-20], and evaporation [21-24]. These methods can be used for pure polymers as well as for composites of polymer [25].

Phase inversion is a process whereby a polymer is transformed in a controlled manner from liquid to a solid state. The process of solidification is very often initiated by the transition from one liquid state into two liquids (liquid-liquid demixing). At a certain stage during demixing , one of the liquid phases (the high polymer concentration phase) will solidify so that a solid matrix is formed. By controlling the initial stage of phase transition the membrane morphology can be controlled, i.e. porous as well as nonporous membranes can be prepared.

Evaporation –induced phase separation (EISP)

The most simple technique for preparing phase inversion membranes is precipitation by solvent evaporation. In this method, a polymer is dissolved in a solvent and the polymer solution is cast on a suitable support, e.g. a glass plate or another kind of support, which be porous (e.g. nonwoven polyester) or nonporous (metal, glass or polymer such as polymethylmethacrylate or Teflon). The solvent is allowed to

evaporate in an inert (e.g. nitrogen) atmosphere, in order to exclude water vapour, allowing a dense homogeneous membrane to be obtained. Instead of casting is also possible to deposit the polymer solution on a substrate by dip coating or by spraying, followed by evaporation.

Vapor- induced phase separation (VIPS)

A cast film, consisting of a polymer and a solvent, is placed in a vapour atmosphere where the vapour phase consists of a nonsolvent saturated with a solvent. The high solvent concentration in the vapour phase prevents the evaporation of solvent from the cast film. Membrane formation occurs because of the penetration (diffusion) of non solvent into the cast film.

Thermally induced phase separation (TIPS)

A solution of polymer in a mixed or single solvent is cooled to enable phase separation to occur. Evaporation of the solvent often allows the formation of a skinned membrane.

Non-solvent induced phase separation (NIPS)

Most commercially available membranes are prepared by immersion precipitation: a polymer solution (polymer plus solvent) is cast on a suitable support and immersed in a coagulation bath containing a non solvent. Precipitation occurs because of the exchange of solvent and non solvent. The membrane structure ultimately obtained results from a combination of mass transfer and phase separation.

All phase inversion processes are based on the same thermodynamically stable solution which is subjected to demixing.

The basic parameter describing the miscibility of two or more components is the free enthalpy of mixing

$$\Delta G_m = \Delta H_m - T\,\Delta S_m$$

where ΔH_m is the enthalpy of mixing and ΔS_m is the entropy of mixing. Two components (polymer/solvent or polymer/polymer) will mix spontaneously if the free enthalpy of mixing is negative ($\Delta G_m < 0$).

The solubility behaviour of polymer solutions differs completely from that of a solution containing low molecular weight components because the entropy of mixing of the long polymeric chains is much lower. Flory and Huggins [26] used a lattice model to describe the entropy of mixing of (polymer) solutions, but it will be not considered here.

2.3.2. Characterisation of membranes

Before describing the membrane characterization methods available and the purpose for which they can be employed, it is important to classify the membranes in two main groups:

porous and non porous membranes.

Characterisation data for porous membranes often give rise to misunderstandings and misinterpretations. It is not unreasonable that it is mainly the size of pore in these membranes that determines which solute can pass or which will be retained. Hence many characterisation methods essentially only determine the pore size and the pore size distributions determined properly, in actual separation processes the membrane performance is mainly controlled by other factors, e.g. concentration, polarisation and fouling. One important, but often not clearly defined variable in the characterisation of porous membranes, is the shape of the pore or its geometry.

Transport through non porous membranes occurs by a solution-diffusion mechanism and separation is achieved either the differences in solubility and/or diffusivity. Hence such membranes cannot be characterised by the methods used for porous membranes.

The determination of the physical properties related to the chemical structure is important for both classes of membranes and it is possible by other methods that allow the determination of surface properties and other physical properties.

Contact angle methods

A drop of liquid sitting on a solid surface represents a powerful, but simple, method to probe surface properties. The phenomenon of the contact angle can be explained as a balance between the force with which the molecules of the liquid (in the drop) are being attracted to each other (a cohesive force) and the attraction of the liquid molecules for the molecules that make up the surface (an adhesive force). An equilibrium is established between these forces, the energy minimum (fig.4). The force balance between the liquid-vapor surface tension (γ_{lv})of a liquid drop and the interfacial tension between a solid and the drop (γ_{sl}), manifested through the contact angle (θ) of the drop with the surface, can be used quantitatively to characterize the energy of the surface (γ_{sv}). The basic relationship describing this force balance is :

$$\gamma_{sv} = \gamma_{sl} + \gamma_{lv} \cos \theta$$

The energy of the surface, which is directly related to its wettability, is a useful parameter that has often correlated strongly with biological interaction [27-30].

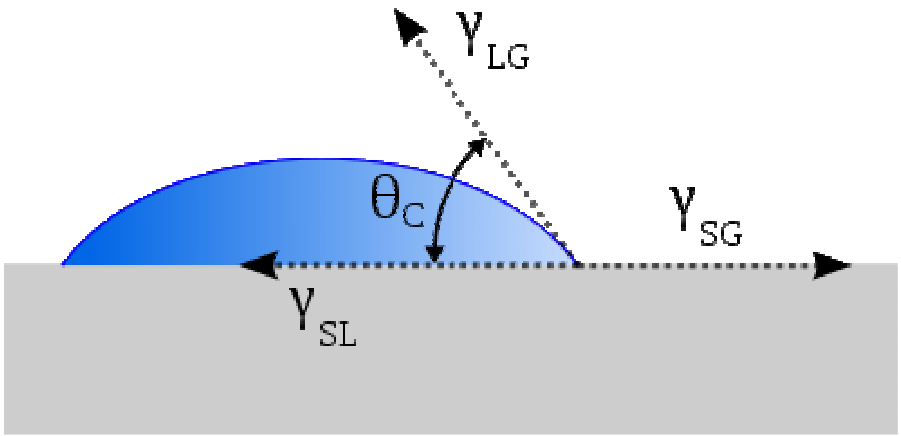

FIGURE 4: THE FORCES INVOLVED IN THE CONTACT ANGLE MEASUREMENT.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) images of surfaces have great resolution and depth of field, with a three-dimensional quality. SEM functions by focusing and rastering a relatively high-energy electron beam (tpically,5-100 keV) on a specimen.

Low-energy secondary electrons are emitted from each spot where the focused electron beam impacts. The measured intensity of the secondary electron emission is a function of the atomic composition of the sample and the geometry of the features under observation. SEM images surfaces by spatially reconstructing on a phosphor screen the intensity of the secondary electron emission. Because of the shallow penetration depth of the low-energy secondary electrons produced by the primary electron beam, only secondary electrons generated near the surface can escape from bulk and be detected. Consequently, SEM is a surface analysis method.

Infrared spectroscopy

Infrared spectroscopy (IR) provides information on the vibrations of atomic and molecular species. It is a standard analytical method that can reveal information on specific chemistry and the orientation of structures. The attenuance total reflectance (ATR) mode of sampling has been used most often in biomaterials studies. The penetration in depth into the sample is 1-5μm. Therefore, ATR is not highly surface sensitive, but observes a broad region near the surface.

Thermal property

Differential scanning calorimetru (DSC) is a method for probing thermal transitions of polymers. A sample cell and a reference cell are supplied energy to varying rates so that the temperatures of two cells remain equal. The temperature is increased, typically at a rate of 10-20 degrees/min over the range of interest, and the energy input required to maintain equality of temperature in two cells is recorded. Plots of energy supplied versus average temperature allow determination of $T_{g,}$ crystallization temperature (T_c), and $T_{m.}$ T_g is taken as the temperature at which one half the change

in heat capacity, ΔC_p, has occurred. The areas under the peaks can be quantitatively related to enthalpic changes.

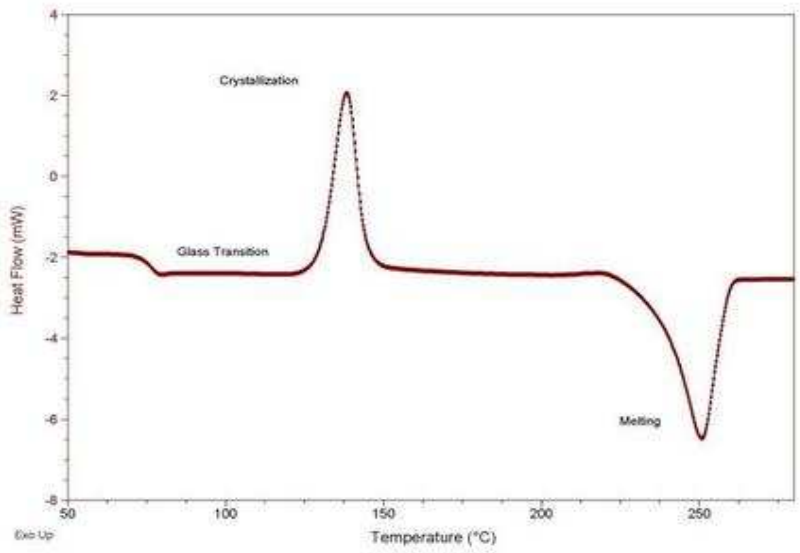

FIGURE 5: DIFFERENTIAL SCANNING CALORIMETRY THERMOGRAM OF A GENERIC POLYMER, SHOWING THE GLASS TANSITION TEMPERATURE, THE CRYSTALLIZATION TEMPERATURE, AND THE MELTING TEMPERATURE OF THE POLYMER SAMPLE.

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Chapter III

Membrane Approaches for Liver and Neuronal Tissue Engineering

3.1 Introduction

Progress in both membrane and cell culture technology has also greatly contributed to the success of artificially engineered tissue. It was demonstrated that polymeric membranes are attractive for their characteristics of selectivity, stability, and biocompatibility in the use of biohybrid systems for cell culture. In particular, semipermeable membranes act as supports for the adhesion of anchorage-dependent cells and allow the specific transport of metabolites and nutrients to cells and the removal of catabolites and specific products [1–3].

In tissue-engineered constructs, the surface and transport properties of the membranes play an important role in the promotion of cell adhesion, proliferation, and viability. The material surface properties, such as chemical composition, hydrophilicity/ hydrophobicity, charge, free energy, and roughness, affect cell adhesion through the modulation of proteins secreted by cells or contained in the physio- logical liquids.

Novel strategies aimed at improving cell– biomaterial interactions have been proposed: the development of new biocompatible and cytocompatible materials and modification of surface chemistry including grafting of functional groups, and immobilization of molecules leaving the bulk properties unaltered.

In this chapter, we review the membrane bioartificial systems developed for tissue engineering. Particular attention is given to the recent achievements in liver and neuronal tissue engineering.

3.2 Membrane for liver tissue regeneration

Each year 30.000 people die of end-stage liver disease in the United States, with an estimated annual cost of 9 billion dollars [4]. Liver transplantation is the only

established successful treatment for end-stage liver disease, and currently there are 100.524 on the waiting list for a donor organ and of those there are 16.005 candidates awaiting a liver transplant (based on United Network for Organ Sharing Organ Procurement and transplantation Network, UNOS OPTN, data as of November 6, 2008). In 2006, there were 6.649 liver transplants performed in United States and 1.935 died while waiting for a liver transplant because of the organ shortage [5]. The European Liver Transplant Registry (ELTR) has cumulated data concerning 75,530 transplantations in 67,848 patients from 136 centres in 23 countries from May 1968 to June 2007 [6]. Cirrhosis is the most frequent indication for transplantation in Europe, followed by cholestatic disease, primary liver tumours and acute hepatic failure [7].

Liver failure is potentially reversible because of liver regeneration [8], so considerable work has been done over many years to develop effective liver support devices. Various non-biological approaches, such as haemodialysis, hemoperfusion, and plasmapheresis have a limited success because of the insufficient replacement of the synthetic and metabolic functions of the liver in these systems [9-10]. On the other hand, extracorporeal biological treatment including whole-liver perfusion, liver slice perfusion and cross haemodialysis, have shown some beneficial results, but they are difficult to implement in a clinical setting [11]. For these reasons, many researchers have developed various extracorporeal bio-hybrid artificial liver (BAL) systems. Generally, a BAL system consists of functional liver cells supported by an artificial cell culture material. In particular, it incorporates hepatocytes into a bioreactor in which the cells are immobilized, cultured and induced to perform the hepatic functions by processing the blood or plasma of liver failure patients. The BAL system acts as a bridge for the patients until a donor organ can available for transplantation or until their liver regeneration [12]. The development of a BAL system involves many design considerations. It must provide: 1) an adhesion support to the cells; 2) an adequate mass transfer of oxygen, nutrients and toxic substances

from blood or plasma of patients to the cell compartments and of proteins, catabolites and other specific compounds produced by cells from the cell compartment to the blood or plasma; 3) immunoprotection of cells and 4) biocompatibility. The BAL devices are classified by the cell source, the type of culture system for the hepatocytes, and the configuration of the bioreactor in Table 1.

References	Bioartificial System	Bioreactor Configuration	Membrane	Cell Source	Cell capacity	Culture technique	Cell position	Level of development
Matsumara et al., 1987 [47]	Kiil dyalizer bioartificial liver	Plate	Cellulose (MWCO 20 kDa)	Primary rabbit hepatocytes	$1x10^{10}$	suspension	Dalysate compartment	First clinical report
Margulis et al., 1989 [48]		Cartridge	Polyvinyl chloride	Porcine hepatocytes	$4x10^{7}$	suspension	Shell	Phase II clinical trials
Sussman et al.1992 [49]	ELAD Amphioxus cell Technology	Hollow Fiber	Cellulose acetate (MWCO=70 kDa)	Human cell line (C3A)	$2x10^{11}$	Aggregates	Shell	Phase I clinical trial
Demetriou et al 1995 [50]	Hepat Assist Circe Biomedical	Hollow Fiber	Polysulphone membranes (Pore size 0.2 μm)	Cryopreserved porcine hepatocytes	$5x10^{9}$	Microcarrier attached irregular aggregates	Shell	Phase II/III clinical trial
Gerlach et al. 1994 [51]	LLS Charite, Humboldt Univ Germany	Hollow Fiber	Polyamide (MWCO=100 kDa) Polyethersulfone (MWCO=80 kDa) Silastic Polypropylene (0.2 μm pore size)	Pig Primary hepatocytes - endothelial cells	$2.5x10^{9}$	Aggregates	Shell	Phase I
Patzer et al 2002 [52]	BLSS Excorp Medical Inc.	Hollow Fiber	Cellulose acetate (MWCO=100kDa)	Porcine Primary hepatocytes	70-120g	Collagen gel entrapped	Shell	phase I/II clinical trials
Flendrig et al 1997 [53]	AMC-BAL Univ. Amsterdam	Spirally wound	Non woven polyester matrix, polypropylene membranes (pore size 0.2 μm)	Pig primary liver cells	$1x10^{10}$	Small aggregates	Shell/on the non-woven Polyester matrix	Phase I
Ding et al., 2003 [54]	BAL TECA Corp.	Hollow Fiber	Polysulfone (MWCO=100 kDa)	Swine hepatocytes	$1x10^{10}$	Aggregates	shell	Phase 0

3.2.1 Cell Source

The choice of the cellular component plays a critical role in the performance of a BAL device. Cells inside must retain their differentiated functions. Primary xenogeneic hepatocytes and human cell lines have been used in the development of BAL.

In particular, do to a lack of human organ availability the current main source of hepatocytes for bioartificial systems is xenogeneic material. Primary porcine hepatocytes, for example, which can be obtained in large quantities, have been widely used as the cell source for hybrid artificial livers. Porcine hepatocytes exhibit biotransformation functions, synthesis of urea, albumin, and other proteins, and are activated by growth factors that also activate human cells [13].

Although the cells are easily obtained in large quantities and demonstrate the same qualities and therapeutic effects of human hepatocytes, this type of cell sources carry the risk of xenogeneic infections and lack of metabolic compatibility [14]. An alternative approach is to use human immortalized hepatocyte cell lines which have the necessary functional and survival characteristics 15-16]. The advantages to using established cell lines include the ability to culture large quantities of cells for an extended period of time and the ability to control the degree of hepatocyte function that is displayed [17-18]. However, because these cells are of cancerous nature it is important to maintain safe handling practices when considering the possibility of using these cells clinically.

Stem cells have been suggested as interesting cell sources. The stem cells, found in sources such as bone marrow, are the most flexible cells in terms that are undetermined in their pathway and express a remarkable ability to differentiate into a desired cell type. Hepatic progenitor cells that are in the liver are often distinguished as primary or small hepatocytes. These cells can differentiate into other functional cells of the liver. Primary hepatocytes are harvested via perfusion and are the

precursor cells to mature hepatocytes. Primary human hepatocytes are very difficult to culture and human cells that are obtained via perfusion do not survive past several divisions [19]. In addition, not only human hepatocytes are difficult to maintain in culture, but also do not perform normal fun]. ctions or undergo differentiation [20]. To sum up, the problems are the limitations in the supply of human hepatocytes, the possible infection in porcine hepatocytes, and tumorigenesis in cell lines and gene-transferred hepatocytes. A preservation method is necessary when using human liver cells as the cell source for a bioartificial liver. The preference for progressive freezing is indicated by a report that compared stepwise freezing with progressive freezing as the freezing method following precultivation of adult liver cells separated from the removed liver [21].

3.2.2 Culture System

Hepatocytes are involved in many important liver functions: blood detoxification, bile secretion, protein, steroid or fat metabolism, vitamin, iron or sugar storage. This multifunctionality implies a great number of biological parameters, which are difficult to reproduce in vitro to keep full functionalities of the hepatocytes. Moreover, primary cultured hepatocytes rapidly lose liver specific functions when maintained under standard in vitro culture conditions. To overcome such limitations in hepatocyte in vitro culture, many strategies to restore and maintain normal hepatic structure and function have been developed, such as the use of protein coated culture dishes [22], collagen sandwich [23], co-culture with other liver-derived or non-liver types of cells [24]. Implantable systems utilizing microcarriers, microcapsules or aggregates have also been developed. Other established culture models have also been able to promote prolonged hepatocyte survival and metabolic function in vitro. These models include the use of porous membranes in a flat configuration, such as the modified polyetheretherketone (PEEK-WC) membrane [25], polyvinyl formal (PVF) resin [26], and poly(D,L-glycolic-co-lactic acid) PGLA foam [27], hepatic multicellular aggregates (spheroids) in suspension culture [28], in microstructured

scaffolds [29] and in pores of porous matrices like polyurethane foam [30] as well as entrapment of hepatic cells in coated alginate beads [31].

3.2.3 Bioreactor

A hybrid liver support device is one of the most complex bioreactor, considering the several functions that liver performs. Its design is to ensure: the rapid detoxification of neural and hepatic toxins; the return of liver specific hepatotrophic factors, as well as liver specific coagulation factors, back into patient's blood; the maintenance of liver cell detoxification and synthetic functions until liver tissue regeneration. On the basis of these considerations, an efficient culture device must be designed considering the following important design criteria: i) to use a sufficient number of differentiated hepatocytes that can maintain the long-term functions; ii) to reduce mass transfer resistances and eliminate substrate limitations so that the device can function at maximum efficiency; iii) to minimize the dead volume in the device thereby reducing plasma dilution effects in the patient. The ideal bioreactor design would maximize mass transfer to the hepatocytes thereby allowing nutrients, including oxygen, and toxins from the patient's blood or plasma to reach the hepatocytes. The treated blood or plasma, including metabolites and synthetic products, would then be returned to the patient's circulation. To achieve this task a large surface area is important for cell adhesion. An ideal device should integrate efficient mass transport, scalability, and maintenance of hepatocyte functions. One of the most promising bioreactors is the membrane bioreactors. Membranes with suitable molecular weight cut-off (MWCO) have been proven to provide an effective immunoisolation barrier to immunocompetent species present in the patient's blood. Thus, xenogenic or allogenic implants may be used without need of immunosuppression therapy. Membranes permit also the transport of nutrients and metabolites to cells and the transport of catabolites and specific metabolic products to blood. In the case of anchorage-dependent cells, they offer high surface area available for cell attachment and culture. In these bioreactor designs mass transfer is

determined by the molecular weight cut-off or pore diameter and occurs by diffusion and/or convection in response to existing transmembrane concentration or pressure gradients. Some bioreactors use membranes with MWCO ranging from 70 to 100 kDa that allow the transport of serum albumin but exclude proteins with high MW such as immunoglobulins and cells (Table 1). The advantage to use these membranes is that to provide immunoprotection. Other bioreactors use microporous membranes with large pore diameter (0.2 □m) that allow the free passage of plasma proteins, toxins and clotting factors between blood or plasma and cells but they exclude the passage of cells (Table 1). The advantage to use a membrane with large pore diameter is to increase the fluid convection in order to improve mass transfer conditions. Polymeric membranes with different morphology and chemical-physical properties have been used in BAL devices [32]. The majority of extracorporeal BAL have used cellulose and polysulfone derivatives (Table 1 and 2). Morphological properties (e.g., pore size, pore size distribution and roughness) and physico-chemical properties (e.g., surface charge, wettability, surface free energy) affect all the adhesion and metabolic functions of hepatocytes [2,33].

However, most of the commercial membranes used for liver cell culture are developed for haemodialysis, which are optimised to be inert with blood proteins and cells. As a result membranes express poor properties regarding cell interactions and functions. Thus, the development of membranes that are able to favour the adhesion and the expression of liver specific functions is quite important for the design of a tissue-engineered liver bioreactor. Several strategies have been proposed to improve the cytocompatibility of membranes including the development of new membranes and surface modification by grafting of functional groups (e.g., COOH, NH_2) or by immobilization of biomolecules such as RGD peptide or galactose that interact with cell receptors [34,35].

Another important issue in bioartificial liver design is the maintenance of sufficient oxygen supply to the hepatocytes. Since hepatocytes are highly metabolic with high

oxygen uptake rates, in order to oxygenate the circulating blood or plasma some devices incorporate an oxygenator in the bioreactor, while other uses an inline oxygenator in the extracorporeal perfusion circuit.

3.2.4 Membrane Bio-Hybrid Artificial Liver (BAL) Systems In Clinical Evaluation

Currently, several BAL devices are in various stage of clinical evaluation and are listed in Table 1. Many of these devices use hollow fiber membranes as supports the cultured hepatocytes and as immunoselective barriers between the plasma of patients and the hepatocytes used in the bioreactor [36].

In 1987 Matsumura et al., reported an early clinical trial of bioartificial liver [37]. The device was developed on the principle of hemodialysis against a suspension of functioning hepatocytes. The liver suspension was placed in a dialysate compartment on one side of a cellulosic semipermeable membrane. The blood flows through a compartment on the opposite side of the membrane (Table 1). Afterwards, one of the first large clinical studies was performed by Margulis et al. in which 20 ml capsules filled with pig hepatocytes in suspension were used [38].

Since the 1990 several BALs were proposed. Sussman and colleagues developed an extracorporeal liver-assist device (ELAD) in which human hepatocyte cell line C3A, which is derived from hepatoblastoma cell line (HepG2) are located outside the hollow fiber and blood flows through the lumen of hollow fibers. A portion of the patient's plasma is ultrafiltrated through a cellulose acetate membrane (70kDa) and is in direct contact with the C3A cells [39]. This device is commercialized by the Amphioxus Cell Technology.

A hollow fiber device that uses cryopreserved porcine hepatocytes attached to a collagen-coated dextran microcarriers that is called Hepatic Assist was developed by Demetriou and coworkers. In this system hepatocytes are loaded into the extracapillary space and patient plasma flows through the capillary lumina of

membranes with pore size of 0.2 μm. This size is sufficiently small to block the passage of whole cells [40]. Plasma first passes through an activated charcoal column and flows through the lumen of the hollow fibers.

A more complex system was proposed by Gerlach et al. The Liver Support System (LSS) or Modular Extracorporeal Liver System (MELS) consists of a bioreactor with four interwoven independent capillary membrane systems that serve different functions. The cells are cultured outer surface and among the capillaries. Each fiber type exhibits a different function: Silastic membranes for oxygen supply and removal of carbon dioxide, polyamide fiber for the plasma inflow, polyethersulfone fiber for the plasma outflow and hydrophilic polypropylene membranes for the sinusoidal endothelial co-culture [41]. With this capillary array decentralised metabolite and gas exchange with small gradients are possible. Due to the independent plasma inflow and plasma outflow compartments, decentralised perfusion of cells between these capillaries is achieved. Additional functions could be integrated into the module.

The bioartificial liver support system (BLSS) is a hollow fiber device that uses porcine hepatocytes embedded in a collagen matrix. This system uses cellulose acetate hollow fibers with a 100 kDa MWCO containing greater than 70 g of primary porcine hepatocytes embedded in a collagen matrix. The patient' s blood is perfused through the capillary lumina [42]. In the circuit a flowing nutrient stream directly perfuses the hepatocytes providing specific nutrients.

The Academic Medical Center Bioartificial Liver (AMC-BAL) developed by Flendrig and coworkers uses a three dimensional, spirally wound, non-woven polyester matrix for hepatocyte attachment with integrated hollow fibers for oxygen delivery to the cells [43]. In contrast with the other systems, the AMC-BAL uses direct contact between the patient's plasma and the matrix attached hepatocyte to improve bidirectional mass transfer.

Another BAL system that is currently in clinical testing is a bioreactor from TECA Corp. in which a polysulfone membrane with MWCO of 100 kDa compartimentalize pig hepatocytes [44].

3.2.5 Membrane BAL system in preclinical and in vitro evaluation

Several BAL systems have been evaluated preclinically *in vitro* experiments and in large animal models of liver failure.

In the Liverx2000 system of Hu and coworkers, the hepatocytes are suspended in a collagen gel and injected into the lumen of hollow fiber with a MWCO of 100kDa and the extracapillary compartment is perfused with a recirculating medium A media flow through the luminal space provides hepatocyte nutrients [45] (Table 2).

References	Bioartificial System	Bioreactor Configuration	Membrane material	Cell Source	Bioreactor cell capacity	Culture Technique	Cell position
Hu et al, 1997 [55]	Liver x2000	Hollow fiber	Polysulfone (MWCO 100 kDa)	Porcine hepatocytes	$1x10^8$	Gel entrapment	Lumen
Shiraha et al., 1996 [56]	BAL	Hollow fiber	Polysulfone (pore size 0.2 μm). Agarose microcapsule	Rat hepatocytes, HepG2	$9x10^7$	Multicellular spheroids	Extrafiber space
Naka et al., 1999 [57]	BLSS	Hollow fiber	Polyethylene (Plasma Flo) (0.3 μm pore size)	Porcine hepatocytes	$5.4x10^9$	Entrapment	Extrafiber space
De Bartolo et al., 2000 [11]	FMB-BAL	Flat	Polyterafluoroethylene and Polycarbonate (pore size 0.2 μm)	Pig hepatocytes	$1x10^{10}$	Sandwich	Between flat membranes
Nagaki et al., 2001 [58]	BAL	Hollow fiber bioreactor	polyolefin fiber 0.4μm pore size	Rat hepatocytes, Hep G2	$2x10^7$	Entrapment	Extrafiber space
Roy et al., 2001 [64]	Flat-plate	Microchannel bioreactor	polyurethane membrane dense	Rat hepatocytes	$2x10^6$	Monolayer	Over the surface
Jasmund et al., 2002 [59]	Oxy-HFB	Crosswise hollow fiber	Polyethylene (pore size 0.2 μm) polypropylene	Pig liver cells	$1\text{-}5x10^9$	Aggregate	Extrafiber space
Mizumoto and Funatsu, 2004 [61]	LLS HALLS	Hollow fiber Multicapillary	polyethylene coated with EVAL hollow fiber polyurethane foam and capillary	Porcine hepatocytes	0.5-100 g	Organoids Spheroids	Extrafiber space
Curcio et al., 2007 [62]	RWMS	Flat	Fluorocarbon dense membrane	Rat hepatocytes	$7.5\text{-}9x10^5$	Spheroids	Over the surface
Schmtmeier et al., 2006 [63]	Minibioreactor	Flat	polyterafluoroethylene membrane dense	Porcine hepatocytes	$6\ x10^6$	Monolayer	Over the surface
Sauer et al., 2004 [65]	Slide reactor	Hollow fiber	polyethersulfone membranes (pore size 0.2 μm)	Human hepatoma cells	$8x10^4$	Aggregates	Between hollow fibers
Pless et al., 2006 [66]	Microchannel bioreactor	Flat-plate	Polyurethane membrane dense	Rat hepatocytes	$2x10^6$	Monolayer	Over the surface
Ostrovidov et al., 2004 [67]	PDMS microbioreactor	flat	polydimethylsiloxane and polyester membrane (pore size 0.4 μm)	Rat hepatocytes	$5\ x10^5$	Monolayer	Over the surface
Ye et al., 2006 [68]	LSS	Hollow fiber	cellulose acetate membrane PMB-30 modified (MWCO 100 kDa)	RTH33 cell line	$2x10^6$	Monolayer	Extracapillary space
De Bartolo et al., 2007 [69]	Multibore fiber bioreactor	Multibore capillary	Modified polyethersulfone (pore size 0.2 μm)	Human hepatocytes	$7.5x10^6$	Small Aggregates	Lumen
Lu et al., 2005 [70]	PVDF-hollow fiber	Hollow fiber	polyvinylidene difluoride (pore size 0.5 μm)	Rat hepatocytes	$5\ x10^7$	Aggregates	Extracapillary space
Memoli et al., 2007 [71]	Membrane bioreactor	Flat	Galactosylated polyethersulfone (pore size 0.1 μm)	Human hepatocytes	$4.7\ x10^7$	Small aggregates	Over the surface

A bioartificial liver support system consisting of hollow fiber cartridge using encapsulated multicellular spheroids of rat hepatocytes was developed by Shiraha et al. The spheroids, formed in a positively charged polystyrene dish were encapsulated into microdroplets of agarose that contained about 9×10^7 rat hepatocytes. The medium was circulated in a closed circuit in which the cartridge was inserted [46].

Several alternative device configurations have advanced to the stage of large animal, preclinical evaluation. Naka et al. have developed a system using primary porcine hepatocytes that is similar to the BLSS. The differences are in the use of microporous polysulphone hollow fiber membranes in the hepatocyte bioreactor and perfusion of plasma through the bioreactor. The system has shown some efficacy in support of ischemic pig liver failure model [47].

The flat membrane bioreactor (FMB) developed by De Bartolo and coworkers consists of primary porcine hepatocytes cultured between semipermeable flat membranes. This is a reproducible model with total hepatectomy in pigs, suitable to test the safety and efficacy of liver support system. Isolated hepatocytes were cultured within an extracellular matrix between oxygen-permeable flat-sheet membranes in the FMB. In particular both sides of the outside shell are constituted of PTFE membranes permeable to oxygen, carbon dioxide and aqueous vapour, which allow direct oxygenation of the cells, adhered to the surface and of the medium overlying the cells. Porcine hepatocytes are maintained in a three-dimensional coculture with nonparenchymal cells. A microporous polycarbonate membrane separates the medium from cell compartment. The FMB maintained stable cell specific functions and is a safe and efficient device [1].

Nagaki and coworkers developed a hybrid liver support system which consists of plasma perfusion through porous hollow fiber modules inoculated with 10 billion of porcine hepatocytes entrapped in a basement membrane matrix, Engelbreth-Holm-Swarm (EHS) gel. This system was applied to pigs with ischemic liver failure. It was demonstrated that the use of a BAL support device in combination with a hollow

fiber module and hepatocytes entrapped in EHS gel has potential advantages for clinical use in patients with hepatic failure [48].

An oxygenating hollow fiber bioreactor (OXY-HFB) BAL system was developed by Jasmund and coworkers and consists of oxygenating and integral heat exchange fibers with a simple design [49]. Primary liver cells are seeded on the surface of the fibers in the extrafiber space. Oxygen requirements are supplied and temperature is controlled via the fibers. Plasma from patient is perfused through an extrafiber space and brought into direct hepatocellular contact.

The liver lobule-like structure module (LLS) BAL system, has many hollow fibers that act as a blood capillary and are regularly arranged close to each other [50]. Hepatocytes are inoculated by a centrifugal force in the outer space of the hollow fibers.

The multicapillary polyurethane foam module (PUF) used as BAL system consists of a cylindrical PUF block with many capillaries in a triangular arrangement to form a flow channel [51]. The hepatocytes in the foam pores formed spheroids with a diameter of 100-150 μm.

Based on the use of a gas permeable membrane a rotating-wall gas-permeable membrane system was developed by Curcio et al., and used for the formation and culture of hepatocyte spheroids. Microgravity conditions were obtained in rotating-wall systems in which hepatocyte aggregates were formed by cells protected from gravitational forces and acceleration. Owing to the high O_2 permeability of the rotating-wall membrane system the viability and functions of cells improved with respect to a polystyrene rotating wall system [52].

Schmitmeier and coworkers developed a new small-scale bioreactor with the hepatic sandwich model. It is of the same dimension as the conventional 24-well cell cultivation plate where the bottom is replaced by the gas-permeable polytetrafluorethylene (PTFE) membrane. Compared to hepatocytes cultured in

conventional systems, primary porcine hepatocytes exhibited stronger liver-specific capacity and remained in a differentiated state in the small-scale bioreactor over a cultivation period of 17 days. This in vitro model could serve as a tool to predict the liver response to newly developed drugs [53].

Pless and coworkers evaluated the primary human liver cells in bioreactor cultures for extracorporeal liver support on the basis of urea production. In particular the long-term course of 47 bioreactor cultures of hepatocytes over a culture period of 21 days was investigated. The bioreactors based on the design developed by Gerlach et al. consist of three interwoven hollow fiber capillary boundless, forming four compartments, and integrated into a polyurethane housing. Two of the boundless are made of hydrophilic polyethersulfone membrane with a pore size of 0.5□m, serving for medium supply during stand-by or for plasma perfusion during clinical application. The third bundle consists of hydrophobic multilaminate hollow fiber membranes and was perfused with a mixture of air and CO2 supplying the cells with oxygen and carbon dioxide [54].

A simple hollow fiber based bioreactor that is suitable for light microscopy was developed by Sauer et al., to evaluate cell-cell and cell-membrane interactions [55]. The SlideReactor offers a cell compartment separated from a medium inflow and outflow compartment. Due to its simple design and the use of materials available in most laboratories, SlideReactor is a simple valuable tool to evaluate the cell-to-cell and cell-to-hollow fiber interaction and enables the comparison of different types or arrangement of hollow fibers, e.g., for use in bioreactor-based extracorporeal liver assist devices, or analysis of the influence of medium supplements on the cell viability and tissue integrity.

Flat plate microchannel bioreactor with an internal membrane oxygenator was designed to improve the oxygen supply to the cells. The hepatocytes are attached to a glass substrate and are in direct contact with the perfusing medium. A polyurethane gas permeable membrane separates the liquid compartment from the oxygenating gas

compartment. This design allows oxygen delivery to the hepatocytes to be decoupled from the medium flow, thereby allowing oxygen delivery and flow to be studied independently. Hepatocytes with oxygen dependent functional heterogeneity may exhibit optimal function into the bioreactor [56].

Ostrovidov and coworkers developed two types of membrane-incorporating microbioreactors to improve the maintenance of primary rat hepatocytes: one with a commercially available polyester membrane and the other with a polydimethylsiloxane (PDMS) membrane. These microbioreactors closely mimic the in vivo liver architecture and revealed to be promising tools towards future application in drug screening or liver tissue engineering [57].

Ye and coworkers presented a bifunctional cellulose acetate (CA) hollow fiber membrane (HFM) bioreactor modified with 2-methacryloyloxyethyl phosphorylcholine (MPC) copolymers (PMB30(MPC-co-n-butyl methacrylate) (BMA) and PMA30 (MPC-co-methacrylic) for preparing a novel liver assist HFM bioreactor. A CA/PMB-PMA30 HFM modified asymmetrically on the inner and outer surface with PMB30 and PMA30 was prepared successfully. The modified HFM has good hemocompatibility and antifouling property on the other surface due to the modification effect of the MPC copolymer [58].

De Bartolo et al., developed a multibore fiber bioreactor as an in vitro liver tissue model to study disease, drugs and therapeutic molecules alternatively to animal experimentation [59]. This bioreactor, owing to the membrane configuration, combines the advantage of having 7 compartments represented by 7 capillaries arranged in one single fiber with high stability and mechanical resistance. Human hepatocytes were cultured in the intraluminal compartment of the multibore fiber bioreactor. The morphological, physico-chemical and transport properties of the multibore fiber membranes favor cell adhesion and ensure a sufficient oxygenation process, nutrient feeding, end-product removal and distribution of fluid molecules inside the cell environment.

Membrane bioreactors using specific adhesive substrates such as galactosylated membranes were developed in order to improve the adhesion and specific functions of liver cells. A galactosylated polyvinylidene difluoride hollow fiber bioreactor provided specific adhesion and showed an enhanced albumin production of rat hepatocytes [60]. Recently a flat bioreactor using galactosylated-polyethersulfone membrane promoted the long-term maintenance of differentiated functions of human hepatocytes outside of the body [61]. This human hepatocyte bioreactor was applied to study IL-6 effects on the production of acute phase proteins and gave evidence that IL-6 down-regulated the gene expression and synthesis of fetuin-A by primary human hepatocytes. The human hepatocyte bioreactor behaves like the *in vivo* liver, reproducing the same hepatic acute-phase response that occurs during the inflammation process.

3.2.5 Novel Membrane Bio-Hybrid System for Liver Regeneration

During the last years several studies demonstrated that semipermeable synthetic membranes can be used for the development of bio-hybrid systems for liver cell cultures. We demonstrated that the morphological and physic-chemical properties of membranes, such as surface free energy parameters, affect cell adhesion and specific metabolic functions of hepatocytes [62, 2]. Polymeric membranes are able to support the long-term maintenance of metabolic and biotransformation functions of isolated human hepatocytes in a bio-hybrid system [63, 25]. Moreover surface modification by plasma process and the immobilization of biomolecule, such as RGD and galactose, on membrane surface improve cell adhesion and the maintenance of differentiated functions [34-35, 64-69].

In the last years the in vitro use of membrane biohybrid systems including bioreactor systems contributed to give important information about the effect of various drugs, such as diclofenac, rofecoxib, paracetamol which effects are not completely known, on the specific functions of human hepatocytes [70-71, 59, 61]. A novel bio-hybrid system constituted by a modified polyethertherketone (PEEK-WC) and polyurethane

(PU) membrane and human hepatocytes was developed by De Bartolo et al., as in vitro system able to reproduce all liver functions to be used for drug testing and toxicity alternatively to animal experimentation. In this system human hepatocytes shown polygonal shape and intercellular junctions with the neighbouring cells forming a structure that is closely the in vivo liver architecture (Fig.1).

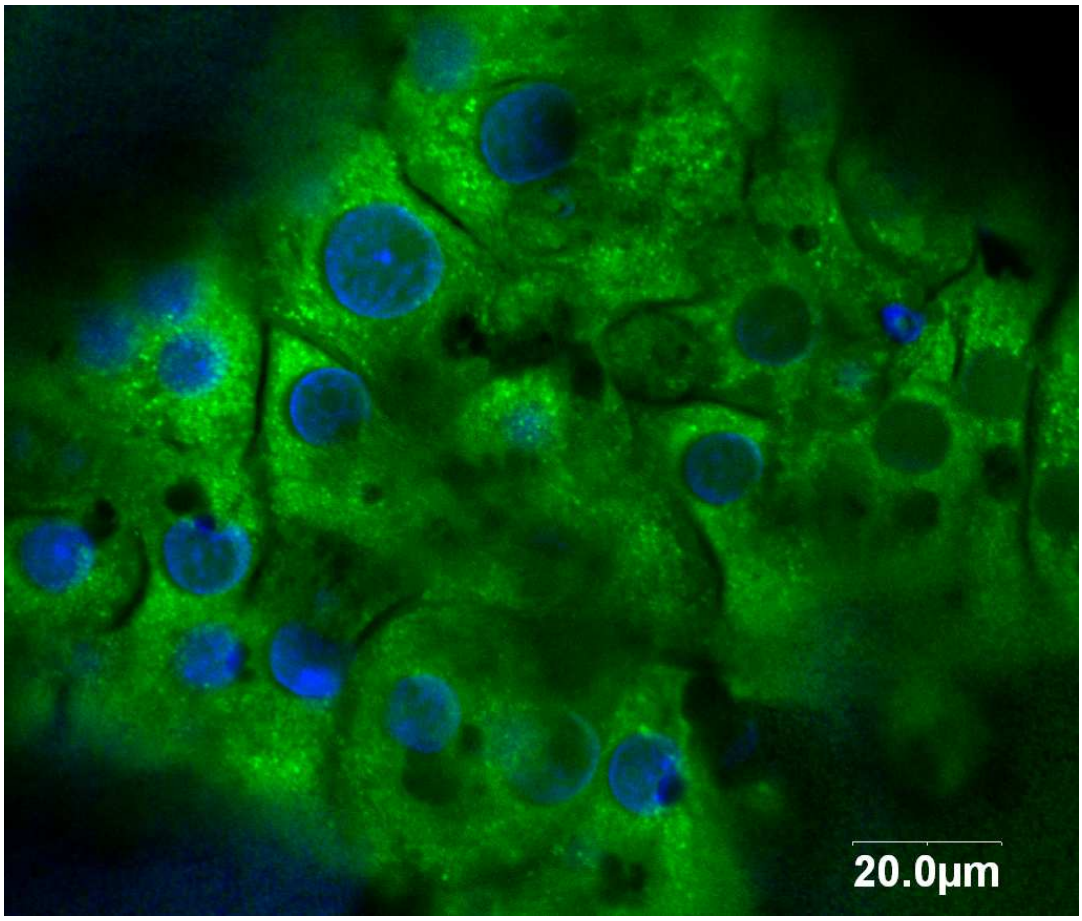

Figure 1 Confocal image of human hepatocytes on PEEK-WC-PU membranes immunostained for actin (green). DAPI (blue) counterstaining was used to detect nuclei. Scale bar 20□m.

This system was used to investigate the effect of a triterpene, ursolic acid (UA), on the specific metabolic functions of human hepatocytes. UA is a pentacyclic triterpene acid which is widely distributed in medical herbs and plants [72] and it possesses anti-bacterial, antiviral, hepatroprotective, immunomodulatory activity [73]. In

particular, the protective effects on the liver cells in the presence of toxic concentration of drug were investigated. Membranes were prepared from a polymeric blend of PEEK-WC or poly(oxa-1,4-phenylene-oxo-1,4-phenylene-oxa-1,4-phenylene-3,3-(isobenzofurane-1,3-dihydro-1-oxo)-diyl-1,4-phenylene) and polyurethane (PU) by inverse phase technique by using the direct immersion-precipitation method. Therefore to obtain information about the mechanism whereby UA reduces drug toxicity, we investigated the effect of UA on the specific functions of human hepatocytes cultured on PEEK-WC-PU membranes in presence of the anti-inflammatory drug Diclofenac (DIC) at a concentration (700 μM) that is known to be toxic. The hepatoprotective effect of UA was evaluated on the liver specific functions such as urea synthesis by using DIC at toxic concentration (700 μM) in the presence of UA (100 μg/ml) (Fig. 2). The ability of human hepatocytes cultured on the PEEK-WC-PU membrane system to synthesize urea was maintained for the whole culture period of 24 days. In presence of DIC the urea synthesis decreased dramatically whereas the administration of the DIC together with UA show less drastic effects on the metabolic functions. It is interesting to note that when the two substances are simultaneously supplied, hepatocytes synthesize urea with a rate which is between each treatment alone.

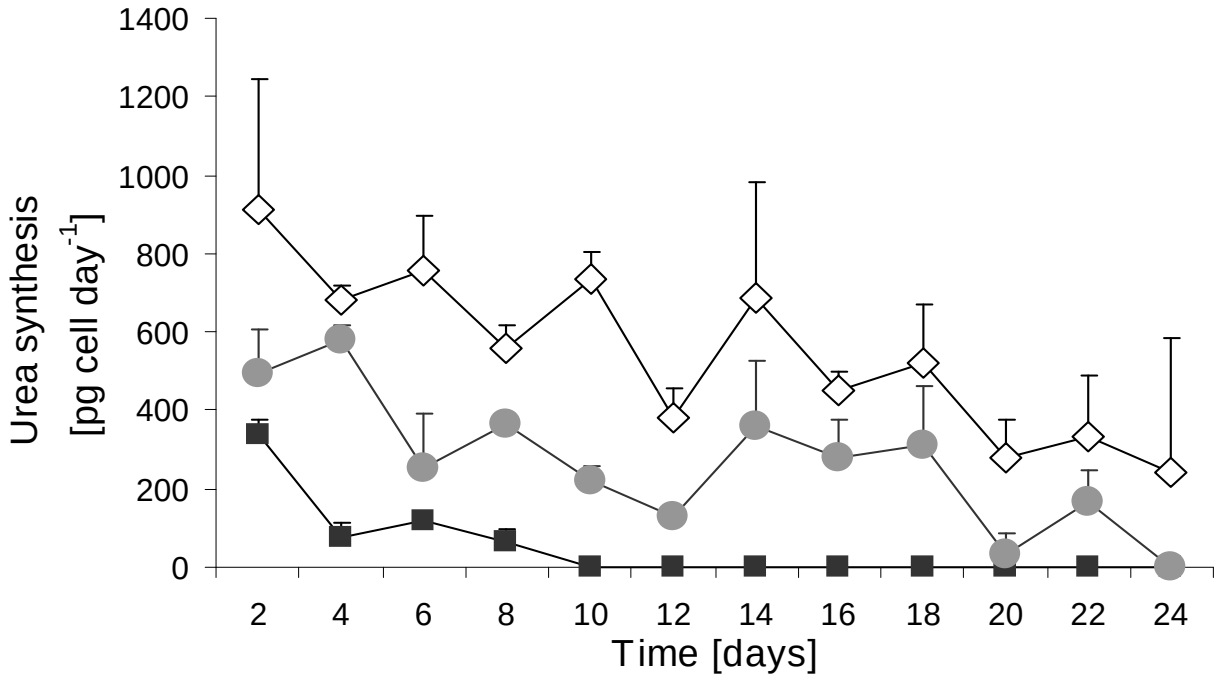

Figure 2 Urea synthesis of human hepatocytes cultured in biohybrid PEEK-WC-PU system for 24 days and treated with: (◊) Ursolic Acid (UA) [100 μg ml^{-1}]; (■) Diclofenac (DIC) [700 μM]; ( ) Ursolic Acid-Diclofenac association (UA+DIC). The values are the mean of six experiments ± standard deviation. On 4th day of culture: * p<0.05 versus DIC; § p<0.05 versus DIC. On 6th day of culture: * p<0.05 versus DIC. On 8th day of culture: * p<0.05 versus DIC; § p<0.05 versus DIC. On 10th day of culture: * p<0.05 versus DIC; § p<0.05 versus DIC; ‡ p<0.05 versus UA+DIC. On 12th day of culture: * p<0.05 versus DIC; ‡ p<0.05 versus UA+DIC. On 16th day of culture: * p<0.05 versus DIC.

These results demonstrated that the biohybrid PEEK-WC-PU system is able to reproduce in vitro the liver functions and therefore it can be used as model system for drug testing and toxicity as well as for studying of metabolic diseases and therapeutic molecules.

3.3 MEMBRANES FOR NEURONAL TISSUE REGENERATION

In the tissue engineering strategies to repair all body part neuronal repair stands out. The nervous system consists of central nervous system (CNS) which includes the brain and the spinal cord and the peripheral nervous system (PNS), which is composed of cranial spinal and autonomic nerves that connect to the CNS. The functional unit of the nervous system is the neuron that has a cell body and dendrites

and axons. The dendrites serve as antenna to receive the signals from the surroundings or other neurons whereas the axon that is longer than dendrite is engaged in transporting impulses from the cell body to dendrites of other neurons through synapses [74]. Electrical impulses can also pass from axon to axon, axon to soma, or from dendrite to dendrite. Injury to the nervous system caused by mechanical, thermal, or ischemic factors can impair various nervous system functions such as memory, cognition language and voluntary movement. The CNS injury may result in death or permanent disability.

In the United States alone, more than 3 million cases of traumatic brain injury are reported annually. These patients include anyone who has fallen, especially someone older than 64 years; a person who has been in a motor vehicle accident; someone with a gunshot wound or violence-related injury; or an adolescent or young adult who has a sports-related injury. Approximately 400,000 Americans suffer from the neurological symptoms associated with multiple sclerosis (MS). Furthermore, over 5 million people in the US and Europe alone suffer from the dementia associated with Alzheimer's disease. About 450,000 people in the United States live with spinal cord injury (one in 670), and there are about 11,000 new spinal cord injuries every year (one in 30,000) [75].

While in the PNS injury the several axons can re-extend and re-innervate with a functional recovery a rare return of damages structures and functions is observed following injuries to the CNS.

Owing to the profound impact of CNS damage, extensive studies have been carried out aimed at facilitating CNS repair. Many strategies have been developed to facilitate the axonal reinnervation and to direct their outgrowth. Various devices using synthetic and biological substrate are being developed as biomaterial bridges as peripheral nerve grafts. In particular membranes in tubular configuration act as guidance channel to protect the regenerating axons in the lumen from the external environment. The membrane reduces the infiltration of fibrous tissue and provides a

conduit for the diffusion of neurotrophic factors and allows the selective transport of molecules between the lumen and the surrounding environment.

Current attempts are focused on seeking new biomaterials, new cell source as well as novel designs of tissue-engineered neuronal bridging devices, to generate safer and more efficacious nervous tissue repairs.

3.3.1 Clinical Approaches for Treating Nerve Injuries

For peripheral nerve injury, one of the current clinical treatments for nerve transaction is surgical end-to-end reconnection, which involves the suturing of individual fascicles within the nerve cable. End-to-end repair, however, is only effective if the nerve ends are directly adjacent and can be reconnected without causing tension. If the injury creates a gap in the nerve, autologous nerve grafts or autografts are used. For longer nerve gaps this approach is not used because any tension introduced in the nerve cable would inhibit the regeneration while an autologous nerve graft from other donor sites is used. This technique has the disadvantage of the loss of function at the donor site and the need for multiple surgeries. There are a few devices that are now FDA approved for relatively short nerve defects, including Integra Neurosciences Type I collagen tube (NeuraGen Nerve Guide) [76] and SaluMedica's SaluBridge Nerve Cuff [77]. However, these treatments are reserved for small defects (several millimeters) and do not address larger peripheral nerve injuries.

For CNS injury, and particularly spinal cord injury, clinical treatment is less promising. If bone fragments exist near the site of injury, then surgery may be performed to reduce any risk of secondary injury. Anti-inflammatory drugs, such as methylprednisone, are often also administered to reduce swelling and secondary injury [78]. Unfortunately, there is currently no treatment available to restore nerve function.

3.3.2 Bioengineering Strategies for Nerve Repair

Bioengineering efforts are focused on creating a permissive environment for regeneration and providing a seamless interface between the CNS and PNS.

Many researchers are presently focusing efforts on creating physical or chemical pathways for regenerating axons. These devices include physical or mechanical guidance cues, cellular components, and biomolecular signals, as reviewed individually below. Future therapies will incorporate multiple cues into unique device that more closely mimic native nerve. They will also

be interactive and programmable, and thus capable of seamless communication with surrounding tissues.

3.3.3 Guidance therapies

The growth of cells and tissue is strongly influenced by environmental cues. In particular, topographical features, such as that created by cells, matrix proteins, and surface texture on biomaterials, influence cell and tissue growth. This phenomenon is termed contact guidance. The physical guidance of axons is a vital component of nerve repair. Since 1960s when Millesi pioneered microsurgical techniques to accurately align nerve fascicles in the direct resection of nerve ends, with improved functional outcomes [79] that is acknowledged the need for physical guidance as an essential element in nerve regeneration. The “nerve guides” or “nerve guidance channels” serve to direct axons sprouting from the proximal nerve end, provide a conduit for the diffusion of growth factors secreted by the injured nerve ends, and reduce the infiltration of scar tissue. Past research in this area has focused either on existing natural or synthetic materials; however, none of the materials studied to date have matched or exceeded the performance of the nerve autograft. As a result, researchers are now focusing on the combination of materials and desired biomolecules to create new composite materials that can actively stimulate nerve regeneration. Matrices are incorporated into the lumen of the guidance channel to enhance the organization of the regeneration environment and provide topographic

guidance cues via aligned textures, and biological guidance cues by patterned biomolecules, to facilitate unidirectional outgrowth of the regenerating axons [80-86]. Cells [87], ECM molecules [88], or growth factors [89] can be incorporated into the tube to assist the axonal sprouting and outgrowth. Among the variety of tubular structures used for guidance channels, semipermeable hollow fiber membranes appear to be a favorable guide to regenerate neuronal tissue. The most promising results were observed with minichannel entubulation device in which Schwann cells were seeded into a semipermeable nondegradable HFM made of a random copolymer of acrylonitrile and vinylchloride (PAN–PVC) with an outer diameter comparable to that of the hemisected part of the right spinal cord [87,90-91]. A large number of both myelinated and non-myelinated regenerating axons were discovered in the midpoint of the channel and some of the regenerating axons were able to traverse the bridge–host interface and renter the host CNS environment. These axons terminated as button like structures in the grey matter. In order to confirm the efficacy of the minichannel design on CNS axon regeneration further evaluations of functional recovery and synaptic reconnections are necessary.

Topographic cues such as the surface microgeometry of the guidance channels, the morphology of the intraluminal matrices within the nerve-bridging device may represent a key element in successful guidance of regenerating axons. In fact, unoriented extracellular matrix materials allow little nerve regeneration [92].

3.3.4 Tissue response to bridging devices

In a tissue-engineered construct the response of tissue is dependent upon the properties of the materials and the cells [93]. Material properties including morphological, structural, mechanical, physico-chemical [94], and electrical properties [95], affect the tissue response. The cells within the construct interact with the material directly via physical contact or indirectly via the diffusion of cell-secreted soluble factors.

In the case of a typical tissue-engineered neuronal bridging device the properties of the material components relevant to the tissue response include the surface microgeometry [96], the molecular weight cut-off [97], the electrical properties [98], possibly the bioactive factors that are loaded and sustainedly released through the channel wall, and the degradation rate for biodegradable materials [99].

Axon guidance by concentration gradients of soluble guidance cues has been extensively studied in vitro. The modification of surface with neuronal trophic factors has been found to elicit neuronal regeneration. The patterning of materials with chemical cues such as ECM proteins (e.g., laminin, fibronectin, RGD peptide has been evaluated in the neurite outgrowth. Neurons from rat brain stem and cortices adhered to surfaces coated with laminin. DRG neurite attachment was found to be dependent on fibronectin strip width (30 μm). Hippocampal neurons adhered to and extended neuritis on the pattern of poly-L-lysine and laminin. Electric fields in the form of voltage gradients have been observed to polarise the nervous system along the rostral-caudal axis during the development (5-18 mV/mm) and direct nerve growth and accelerate wound healing in the rat cornea (+40 mV/0.5mm) [100]. Cues in three-dimensions such as matrigel, collagen, as well as polymer synthetic polymer-based scaffolds support longer neuritis than in bidimensional culture systems. Topographical cues influence nerve growth and regeneration. The influence of topographical features on neuronal cell adhesion and differentiation has been studied by using patterned adhesive areas that provide only a fraction of the surface for cell adhesion while the rest is cell-repellent or by using contact guidance cues in combination with also nerve growth factors or electric field [101-103]. Neurons have the ability to respond to topographical features in their microenvironment and they have been shown to adhere, migrate, and orient their axons to navigate surface features such as grooves in substrates and the micro-and nanoscales. Topographical guidance of neurite outgrowth has been explored in vitro with well defined micropatterned substrates. Topographic guidance of neuritis outgrowth has been

explored in vitro with culture substrate containing etches, microchannels, nanotubes or microgrooves [104-108]. Mahoney et al., studied the effects of microchannels of 20 to 60 μm in width and 11 μm in depth on PC12 cell cultures [109]. Neurites were directed along the axis of the grooves with microchannels of 20 to 30 μm being most effective at neurite directions. Other Authors found an optimal surface with roughness in the range of 50 nm for the attachment of primary neurons isolated from the substantia nigra [110].

De Bartolo et al., shown that hippocampal neurons exhibited a different morphology in response to varying the properties of the membrane surface [111]. Indeed, cells grown on the smoother membranes and namely Fluorocarbon (FC) and polyethersulfone (PES) membranes displayed a large number of neurites with consequent formation of bundles. The density of axonal network increases the neuritis become more elaborate and highly branched on the smoother surfaces (Fig.3).

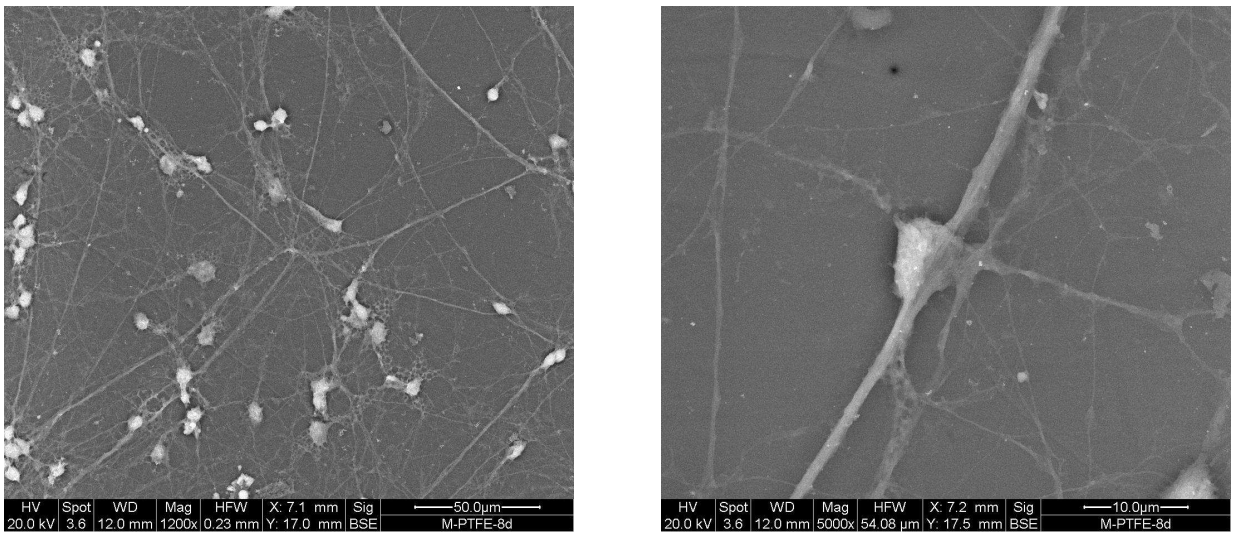

Figure 3 Micrographs of hippocampal neurons after 8 days of culture on modified fluorocarbon membrane (FC); a) and b) different magnification.

As a consequence while a very complex network was formed on these membranes, cells tend to, instead, form aggregates and most of the processes are developed inside the pores of the membranes when rougher PEEK-WC surfaces were used. Aebischer et al., [96] observed that smooth inner walls of polymeric channels induced the formation of discrete nerve cables with a number of myelinated axons within an organized fibrin matrix in contrast with rough inner walls that elicited the formation of scattered nerve fascicles in an organized fibrin matrix.

Zhang et al., have fabricated HFMs with unidirectional aligned grooves parallel to the hollow fiber long axis on the inner surfaces from both nondegradable and biodegradable natural and synthetic polymers. HFM guidance channels with aligned grooves on the inner surfaces may allow enhanced orientation and directional outgrowth of regenerating axons [112]. Both surface and transport properties of the membranes play an important role in the neuronal regeneration: the surface microgeometry of the membrane inner wall affects the orientation of the axonal growth; the molecular weight cut-off of the membrane affects the axonal regeneration by governing the mass transfer of molecules between the controlled regenerating environment within the channel lumen and the external environment [97]. In table 3 are reported the main studies with neuronal cells using membrane systems.

REFERENCE	MEMBRANE	CONFIGURATION	MATRICES	MEMBRANE PROPERTIES	CELLS	EFFECT
Li et al., 1999 [129]	Polyacrylonitrile polyvinylchloride copolymer	Hollow fiber	Macroporous cellulosic microcarriers	Hydrogel	PC12 L-dopa secreting and C2C12 CNTF secreting	Cell proliferation, dopa and CNTF secretion
Manwaring et al., 2001 [124]	Glass, CA, PES, PAN-PVC, PS, PP, PEVAC	Flat	-	Contact angle ranging from 35 to 95°	Dorsal root ganglion and cerebellar granule neurons	Cell proliferation on CA, PAN-PVC, PEVAC and glass
Broadhead et al., 2002 [130]	Copolymer of acrylonitrile and vinylchloride	Hollow fiber	-	Cut-off 40,150 kDa	PC12	Proliferation and maintanance of viability
Lee et al., 2003 [125]	polyethylene	flat	Corona treated	Wettability gradient surfaces	PC-12	Longer neurites on surface with contact angle of 55°
Zhang et al., 2005 [122]	Aromatic polyether based polyurethane, Poly DL-lactide-coglicolide	Hollow fiber	Poly-L-lysine and laminin	Grooves on the lumen of width width of 38.5-91 μm	Dorsal root ganglion	High rate of neurite outgrowth on textured inner surface
Young et al., 2005 [123]	Polyvinyl alcohol and polyethylene-co-vinyl alcohol	flat	-	Dense structure	Cerebral cortical stem cells	Differentiation in neurons/strocytes and porlifertaion
Lopez et al., 2006 [127]	silicon	flat	Laminin and collagen	Pore size ranging from 20 to 50 nm	PC-12	The modification of collagen increase cell survival and functionality
Chang et al., 2007 [126]	Polyethyleneimine-polyethylenevinylalcohol	flat	-	Contact angle 23.2-84.9°	Rat cerebellar granule neurons	Increased cell viability on surface with 57.8°
De Bartolo et al., 2008 [121]	Fluorocarbon, polyethersulfone, modified polyetheretherketone,	flat	Poly-L-lysine	Roughness ranging from 6 to 200 nm	Hippocampal neurons	Surface with Ra up to 50 nm favoured the formation of longer neurites and BDNF secretion

The molecular weight cut-off of the membrane can be adjusted to allow diffusion of nutrients, waste products, and the external humoral factors that are beneficial to regeneration across the membrane– tissue interface, excluding the transport of immunocompetent species and molecules. Polyvinyl alcohol (PVA) and polyethylene-co-vinylalcohol (EVAL) membranes were used for culture of neural stem cells isolated from embryonic cerebral cortex. Single neural stem cells seemed to remain dormant on the EVAL. Conversely the development of cell clusters at low density allowed the differentiation of cells into neurons and astrocytes [113]. The physico-chemical properties of the membrane surface are also important in the interactions with cells. Manwaring et al., cultured cells derived from meningeal tissue on surfaces with different wettability: PAN-PVC, polyethylene vinyl acetate (PEVAC), polypropylene (PP) polystyrene (PS) and Tecoflex (TECO). The hydrophilic materials supported the highest level of cell attachment while the hydrophobic materials supported less cell adhesion [114]. Other studies demonstrated that PC-12 cells are a differentiated neuronal phenotype with a long neurite on polyethylene surface with moderate wettability [115].

Chang et al., studied the effect of membranes made of a polymeric blend of polyethyleneimine (PEI) and EVAL with different concentration of PEI on the behaviour of granule neuronal cells. The addition of PEI at increasing concentration resulted in a change of wettability properties of the membranes with contact angle ranging from 23 to 85°.An increased cell viability was observed on membrane surface with 57.8° [116].

In the understanding the interactions between microfabricated synthetic interfaces and cells Lopez et al., investigated microfabricated nanoporous silicon membranes modified with collagen and laminin on the survival, proliferation and differentiation of PC-12 cells. The modification of the membrane with collagen was important to improve the adhesion of cells [117].

The tubular membrane can be engineered to allow the adsorption of the bioactive factors to the channel walls during the fabrication process, which could be released within the lumen to favour the nerve regeneration. Previous studies reported enhanced nerve regeneration in vivo under the effect of growth factors or neurotrophic factors released from the walls of the guidance channels [118].

Both non biodegradable such as silicon, polyethylene, polyvinylchloride, polyvinylidenedifluoride, polytetrafluoroethylene, and biodegradable materials as collagen, chitosan, polycaprolactone, polyglycolic acid have been used to realize tube as nerve guidance channels for repairing of transected nerves. Particularly semipermeable hollow fiber membranes seeded with fetal spinal cord cells have been shown to markedly promote axonal growth across a gap in adult rat spinal cord. Zhang et al., used semipermeable hollow fiber membranes with highly aligned textures on the inner surface in order to promote the dorsal root ganglion regeneration. Aromatic polyether polyurethane was used as non degradable and poly-DL-lactide-coglycolide was emplyoed as a degradable polymer. The presence of groove with heights of 38.5 to 91 μm allowed the regeneration of neuritis that were aligned and parallel to the direction of the grooves [86].

Asymmetric hollow fiber membranes of polyacrylonitrile and polyvinylchloride (PAN-PVC) used to encapsulate PC12 cells secreting dopa and C2C12 secreting ciliar neurotrophic factor (CNTF) supported the proliferation of cells and the secretion of specific factors [119]. PAN-PVC membranes with structural properties have been used for the PC12 cells by Broadhead et al. [120]. The encapsulated cell biomass, the number of proliferating cells, and the quantity of dopamine released increased as a function of increasing hollow fiber membrane diffusive permeability. In this respect the membrane permeability plays a dual role by regulating the size of the cell mass that generates the product and in its ability to restrict the diffusion of the product across the membrane into the surrounding environment. Unlike biomass size the percentage of viable cells was independent on the membrane permeability.

The use of biodegradable materials in guidance channels offer the advantage timely to disappear from the implantation site without an additional surgical intervention once the regeneration process is completed and further allowing the connection of the regenerated axons with host circuitry. Important characteristics of a biodegradable guidance channel include minimal tissue response following implantation, in vivo degradation at a rate that matches with the regeneration process, to non-toxic and readily excreted degradation products, and the absence of toxic residual chemicals that may be contained in their preparation [99].

For most of the biodegradable materials that are well tolerated in the CNS, such as polylactic, polyglycolic acid, and their various copolymers [121-123], polyanhydrides [124], hydrogels [125], and poly(phosphoester) [126], the tissue inflammatory response generally becomes stabilized and resolved as a function of material degradation. Biodegradable materials formulated into microspheres were designed for controlled drug delivery at a CNS tissue site.

3.3.5 Membranes Used In Vivo Neuronal Regeneration

Synthetic hollow fiber membranes can be used to repair the nerve transaction. Transplantation of porous tubes in poly(2hydroxyethyl methacrylate-co-methyl methacrylate) was performed by Reynolds et al., following a spinal cord transaction surgery in order to improve locomotor functions in rat [127] (table 4).

REFERENCE	MEMBRANE	CONFIGURATION	MATRICES	MEMBRANE PROPERTIES	CELLS	EFFECT
Benvensite et al., 1987 [139]	Diaflo	Microdialysis tube	-	50 kDa		Cellular reaction to implant
Winn et al., 1989 [140]	PAN-PVC	Capsules	-		Giant cells	Immunoisolation form xenograft
Aebischer et al., 1989 [107]	Polysulfone	Tubes	-	MWCO 10 and 100 kDa	Sciatic nerve	Regeneration of peripheral nerve with 10 kDa MW cut-off membrane
Li et al., 1999 [129]	Polyacrylonitrile polyvinylchloride copolymer	Hollow fiber	Macroporous cellulosic microcarriers	hydrogel	PC12 L-dopa secreting and C2C12 CNTF secreting	Cell proliferation in vitro and in vivo and release of L-dopa and CNTF
Sajadi et al., 2006 [141]	Polyethersulfone	Fiber	Polyvinylalcohol	Inner diameter 500 μm	MDX12 secreting GDNF	Regeneration of nigrostrial dopaminergic fibers
Reynolds et al., 2008 [137]	Poly-2-hydroxyethyl methacrylate-co-methyl methacrylate	Tube	-	Dense structure		Improvement of locomotion function after spinal transection
Xue et al., 2001 [142]	Alginate-polylysine-alginate	Microcapsules	Polylysine	Diameter 100-300μm	Bovine chromaffin cells	Reversion of behavioral deficits in hemiparkinsonian rats
Kim et al., 2004 [143]	alginate	Microcapsule	Poly-L-lysine	Diameter 100-300μm	Chromaffin cells	Maintenance of cell viability after implantationand analgesic effect

Semipermeable membranes are also used for the cell encapsulation in order to immunoisolate the cells from the host by being permeable to molecules smaller than certain sizes, but restricting the passage of larger molecules such as antibodies and complement components from entering the membrane lumen and interacting directly with the encapsulated cells [128]. One of the applications of hollow fiber membranes with encapsulated cells regards the implantation in the CNS for treatment of Parkinson ’s disease. Parkinson's disease is a neurological disease characterised by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta, which are important to motor control. A treatment strategy under development involves the implantation of encapsulated dopamine-secreting cells, with the purpose being to alleviate at least some of the symptoms of Parkinson's disease through the targeted delivery of dopamine. There also are other nervous system disorders where the delivery of a missing factor(s), e.g., neurotransmitters, neurotrophic factors, or enzymes, might compensate for a disease-caused deficiency and thus alleviate symptoms. Although approaches such as the use of pumps or slow-release polymer systems could be employed, the use of encapsulated secretory cells is a particularly attractive one for chronic implant therapies since the supply will last as long as the cells survive. There are two methods of encapsulation micro-and macroencapsulation [128]. For macroencapsulation, cells are usually suspended in a matrix within a hollow fiber membrane. The open ends of the hollow fiber are sealed thereby forming a capsule within which cells reside. In this case the relatively thick fiber membrane represents a large diffusion distance for the release of catecholamine. This limit can be overcome by microcapsule. The thin membrane and spherical shape result in a high surface-to-volume ratio that facilitates transmembrane diffusion and enhances cell viability. A disadvantage of the microcapsules is that they are fragile and cannot be retrieved easily.

One of the first studies concerning the implantation of hollow fiber membranes is that of Benvensite, which implanted a Diaflo hollow fiber membrane microdialysis tube

in the rat hippocampus and studied the cellular reaction. Hypertrophic astrocyte processes invaded the spongy fiber wall three days after and late collagen deposits and occasional granuloma were formed. This study represents an initial attempt toward understanding the CNS tissue response to an HFM device [129]. Afterwards, Winn et al. [130], encapsulated cells in a PAN–PVC HFM implanted into the adult male rat right parietal cortex. A layer of reactive astrocytes mixed with other inflammatory cells including macrophages and microglia enveloped the membrane with some of the cells penetrated into the fiber wall. However, the response was benign due to a consistent absence of necrosis at or around the implant–brain interface over time.

Semipermeable polysulfone tubular membranes with MWO ranging from 100 to 1000 kDa were used in transacted hamster sciatic nerve model to support nerve regeneration in the absence of a distal nerve stump by Aebischer et al. [97]. The MWCO of the tubular membrane affect the outcome of regeneration. Only the membranes with MWCO of 100 kDa supported the regeneration of well differentiated peripheral nervous tissue containing a significant number of myleinated axons.

Li et al., [119] used asymmetric hollow fibers membranes of polyacrylonitrile and polyvinyl chloride copolymer (PAN-PVC) to encapsulate PC-12 cells secreting L-dopa and dopamine attached to macroporous cellulosic microcarriers. PC-12 cells on microcarriers were embedded in PEO or agarose within hollow fiber devices. Devices were implanted into rodent striatum for 4 weeks and assayed for catecholamine release. Proliferation control is attained by embedding cell containing microcarriers in nonmitogenic hydrogels.

Glial cell line derived neurotrophic factor have been encapsulated in hollow fiber membranes of polyethersulfone previously filled with a polyvinylacohol cylindrical matrix and the fiber ends sealed with acrylic based glue. The implantation of encapsulated cells by PES fibers in animal models of Parkinson’s disease [131] leads

to improvement of movement associated with straiatal reinnervation of dopaminergic fibers.

Microcapsules made of Alginate-polylysine-alginate have been used by Xue et al. [132], to encapsulate bovine chromaffin cells that have been implanted into the brain of hemiparkinsonian rats. The results of this study show great promise in the application adrenal autograft tissue for the treatment of Parkinson's disease. Bovine chromaffin cells have been also encapsulated in microcapsule of alginate with 100-300 □m diameter covered by poly-L-lysine and implanted in subarachnoid space of rats. The microcapsules reduced the symptoms of pain. The cells were morphologically normal and retained their functionality [133].

3.4 CONCLUSIONS

With the recent advances in the field of biomaterials particularly in the membrane systems there is much promise of working towards a functional tissue engineered constructs. Many strategies are being developed to realize tailored synthetic and biodegradable membrane systems that are compatible with human cells and tissues. The preclinical development stage of some of these membranes systems demonstrates their potentiality in the tissue engineering field. Bioartificial membrane systems could have a role not only in the replacement of injured organ or tissue but could accelerate the development of new drugs that may cure patients alternatively to animal experimentation. Many problems encountered with testing potential drugs can be overcome and redesigned on a quicker time scale and at lower cost utilizing a system that can effectively act as a functioning liver. Considering the multidisciplinary nature of the tissue engineering it is important for clinicians, basic scientists and engineers to collaborate and to explore all areas of possibilities in order to realize the new advancements towards the creation of an entire organ.

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Chapter IV

Biodegradable and synthetic membranes for the expansion and functional differentiation of rat embryonic liver cells

4.1 Introduction

The ability to expand hepatic stem cells or embryonic liver cells is desired to generate cells for liver tissue engineering in clinical and pharmaceutical applications. Conventional methods for expanding hepatic stem cells or progenitor cells comprised polystyrene culture dishes and component of extracellular matrix such as collagen [1]. Alternatively biocompatible and biodegradable materials have been proposed to support cells and promote their differentiation and proliferation towards the formation of a liver tissue [2].

Natural polymers such as chitosan are advantageous for liver tissue engineering because of their similar structure of glycosaminoglycans, which are components of the liver extracellular matrix. Chitosan is the N-deacetylated derivative of chitin and is naturally degraded by body enzymes. In addition, its reactive amino and hydroxyl groups provides many possibilities for covalent and ionic modifications which allow extensive adjustment of mechanical and biological properties. Due to its excellent biocompatibility and bioadsorbility chitosan has been used in several biomedical applications (e.g., skin, bone, cartilage, blood vessels, haemodialysis, nerve and liver) [3-8]. Scaffolds constituted of chitosan crosslinked with other biomolecules have been applied in the culture of liver cell line and primary hepatocytes [9-10]. Chitosan in membrane configuration modified with biomolecules have been used for culture of fibroblast cell line and in the form of galactosylated scaffold has been applied to induce the formation of rat hepatocyte aggregates [11-12].

Membranes prepared from chitosan could act as synthetic extracellular matrix combining the advantageous properties of the polymer (i.e. biocompatibility, biodegradability and biofunctionalities) with those of membranes such as permeability, selectivity and well-defined geometry [13-14]. In fact membranes would allow the organization of the cells into a three-dimensional architecture, providing mechanical integrity and a space for the diffusion of nutrients and metabolites to and from cells [15-16].

Synthetic polymeric membranes can be used also in the development of in vitro engineered constructs to reproduce a physiological tissue model for studying drugs and metabolic diseases. Modified polyetheretherketone (PEEK-WC) membranes have good stability, permeability and biocompatibility properties, as previous studies with rat hepatocyte culture demonstrated [17]. They can easily be mass produced with modulated morphological and physico-chemical properties for specific applications but are not degradable and for this can be used only in vitro systems.

Herein we examine the expansion and the expression of differentiated functions of rat embryonic liver cells on chitosan and PEEK-WC membranes in comparison with traditional substrates such as collagen and polystyrene culture dishes (PSCD). Rat embryonic liver cells (17 day embryo) was used in this study as alternative model of human liver progenitor cells [18-19] since using cells from the foetal human liver is limited by a major ethical issue. Embryonic liver cells have many advantages over primary hepatocytes for proliferation in vitro to transplantation in vivo [20].

4.2 Materials and methods

4.2.1 Membrane preparation

Chitosan membranes were prepared by dissolving 1% (w/v) of chitosan (Sigma Co., Milan Italy) in acetic acid solution (2%, v/v). Then polyethylene glycol (PEG) with MW of 6000 D (Merck- Schuchardt) was added to the chitosan solution in 4:1 ratio and stirred for 2 h. The solution was cast on glass plate by means of commercial knife (Elcometer, Adjustable Bird Appl 0-250 μm) and dried at room temperature.

The membrane was immersed in a solution of 1% NaOH after drying. Finally, the membrane was washed in deionised distilled water for the test.

Synthetic membranes were prepared from the modified PEEK-WC or poly(oxa-1,4-phenylene-oxo-1,4-phenylene-oxa-1,4-phenylene-3,3-(isobenzofurane-1,3-dihydro-1-oxo)-diyl-1,4-phenylene) (patented by Zhang et al. [21] and provided by the Institute of Applied Chemistry, Cancun, China) by means of the inverse phase technique by using the direct immersion-precipitation method as previously described [17]. The PEEK-WC is obtained by polycondensation reaction between 4,4-dichlorobenzophenone and phenolphthalein [22].

4.2.2 Membrane Characterization

The membranes were characterised by Fourier Transformed Infrared Radiation (FT-IR) analysis in order to identify the specific chemical groups of the polymers. The spectra were recorded on a Perkin Elmer 1300 spectrometer.

The wettability properties of all membranes were characterised by using the water contact angle measurements by sessile drop methods and water sorption at ambient temperature by using CAM 200 contact angle meter (KSV Instruments LTD, Helsinki, Finland). Results are the mean of ten measurements of different regions of the sample surface. All measurements were repeated for six times.

Swelling and dissolution tests were performed for chitosan membranes. Membrane samples (1 mm×1 mm) were weighted and then put in 1 ml phosphate buffered saline (PBS) at 37°C. Swollen membranes were drawn at various times and were weighted again. The swelling index (SI) was calculated as: $\%SI = \frac{Ws - Wi}{Wi} \times 100$ where Wi and Ws are the sample weights before and after incubation in PBS respectively. After drying at 37°C for 48 h samples were weighted again and the solubility percentage was calculated as: $\%S = \frac{Wi - Wd}{Wi} \times 100$ where Wd is the dried sample weights after dissolution test. Each test consisted of four replicate measurements.

4.2.3 Cell culture

The rat embryonic liver cells (17 days embryonic liver of Japanese albino rat) (RLC-18) were obtained from the DSMZ (Braunschweig, Germany). Cells were maintained in RPMI medium containing 10% FCS (Biochrom AG, Berlin, Germany), l-glutamine, penicillin and streptomycin (Biochrom AG, Berlin, Germany) at 37 °C in a humidified CO_2 incubator (95% air 5% CO_2) and subcultured twice a week by using trypsin (0.05 %)/EDTA (0.025 %) solution (Carl Roth GmbH, Karlsruhe, Germany) (Sigma-Aldrich, Munich, Germany). The cells were cultured at 6 passages on the chitosan and PEEK-WC membranes, and on collagen I (BD Biosciences, Germany) and PSCDs. Cells were cultured for up to 24 days, the medium was changed every 48 h.

Experiments were performed in presence of Diazepam 10 □g/ml in the culture medium to evaluate the ability of cells to perform drug biotransformation functions, in particular the elimination of diazepam and the formation of its metabolites.

The morphology of the cells cultured on the different substrates was assessed by means of Scanning Electron Microscopy (SEM) and Laser Confocal Scanning Microscopy (LCSM).

Liver-specific cellular functions were investigated in terms of albumin production and urea synthesis.

4.2.4 Cell fixation for SEM

Cells cultured on membranes, collagen and PSCD were prepared on day 7 and 16 for SEM analysis by fixation in 3% glutaraldehyde and 1% formaldehyde in PBS, followed by post-fixation in 1% osmium tetroxide and progressive ethanol dehydration.

4.2.5 Cell staining for LCSM

The morphological behaviour of embryonic cells cultured on the investigated substrates was investigated at 7 and 16 days of culture with Laser Confocal Scanning

Microscopy (LCSM) after cytoskeleton and ECM protein immunostaining. Samples were rinsed with PBS, fixed for 15 min in 3% paraformaldehyde in PBS at room temperature (RT), permeabilized for 5 min with 0.5 % Triton-X100 and saturated for 15 min with 2% Normal Donkey Serum (NDS).

To visualize vinculin, a mouse monoclonal antibody raised against human vinculin (Santa Cruz Biotechnology, Santa Cruz, CA) and a Cy^{TM}3-conjugated AffiniPure donkey anti-mouse IgG (Jackson ImmunoResearch Europe Ltd, Cambridge, UK) was used. Actin was stained with phalloidin Alexa 488 conjugated (Molecular Probes, Inc, Eugene, OR). All primary and secondary antibodies were incubated for 2 and 1.5 hours at RT, respectively. Counterstaining for nuclei was performed with DAPI 0.2 µg/ml (Molecular Probes Inc, Eugene, OR) incubated for 30 min. Finally samples were rinsed, mounted and observed with a Laser Confocal Scanning Biological Microscope (Fluoview FV300, Olympus Italia).

4.2.6 Biochemical assays

Samples from the culture medium were collected in pre-chilled tubes and stored at -20°C until assayed. Albumin secretion was measured with the immunoenzymatic ELISA method. 96 well plates were coated with chromatographically purified rat albumin (Sigma, Milan, Italy) 50 µg/ml and left overnight at 4°C. After 4 rinses, 100 µl of cell culture supernatant were added to the wells and incubated overnight at 4°C with 100 µl of anti rat albumin monoclonal antibody conjugated with horseradish peroxidase (Bethyl Laboratories, Inc., USA). After 4 rinses, the substrate buffer containing Tetramethylbenzidine and H_2O_2 (Sigma, Milan, Italy) was added for 7 min and the reaction was stopped with 100 µl of 8 N H_2SO_4. Absorbance was measured at 450 nm using a Multiskan Ex (Thermo Lab Systems).

The urea concentration was determined by the quantitative colorimetric urea assay kit QuantiChrom^{TM} (Gentaur, Brussels, Belgium).

The statistical significance of the experimental results was established according to the ANOVA followed by Bonferroni, as post hoc test ($p < 0.05$).

4.2.7 HPLC analysis of diazepam and metabolites

Embryonic cells were incubated with 10 μg/ml diazepam; HPLC was used to monitor the biotransformation of diazepam [23] in its metabolites. Aliquots of the culture medium were alkalinized with 20% of 4 M NaOH, precipitated with isopropanol (1:10) and extracted with ethyl acetate (5:1) by gentle rocking for 10 min and subsequent centrifugation at 200 *g* for 15 min at RT. The ethyl acetate phase was then evaporated and dried under vacuum, and the pellet dissolved in 96 μl mobile phase consisting of acetonitrile/methanol/0.04%triethylamine pH 7.04 at proportion of 25/35/40 respectively. The samples were HPLC analysed using a C_{18}-RP Purosphere Star 5 μm, 250x4,6 mm column, equipped with a precolumn (Merck KGaA, Darmstadt, Germany). The sample injection volume was 20 μl. The mobile phase was delivered at 0.8 ml/min and the column was operated at ambient temperature. Effluents were monitored with a UV detector at 236 nm. Besides diazepam its metabolites temazepam, oxazepam and nordiazepam were detected. For all substances calibration curves were regularly run between 10 ng/ml and 10 μg/ml.

4.2.8 Western Blot

Cells were pelletted by centrifugation and the proteins extracted using the phenol extraction method described by Wang et al. [24].

The final pellet was dried and dissolved in Laemmli sample buffer for 1 DE separation. For 1 D separation we used the already precasted gels from BIO-RAD (4-20% polyacrylamide).

The pellet was incubated overnight in loading buffer. After denaturation at 95°C for 3 min, proteins were resolved under constant 120V in a BIO-RAD mini protean tetra system (BIO RAD) until bromophenol blue reached the bottom of the gel.

1 DE (mono-dimensional) gels were processed for western blots and then the protein blots were blocked overnight with 3% bovine serum albumin in TBST buffer (20 mM Tris-HCL, pH 7.6, 0.8% NaCl, 0.1% Tween 20) and incubated with monoclonal antibody against albumin (Santa Cruz Biotechnology, 1:500), cytokeratin 18 (CK18) (Santa Cruz Biotechnology 1:200) and Alpha-fetoprotein (AFP) (Santa Cruz Biotechnology 1:200) [25].

Blots were washed and incubated with a goat anti-mouse horseradish peroxidase-conjugated antibody (1:1500, BIO-RAD). The detection was performed with Opti-4 CN (4-chloro-1 naphtol) substrate and detection kits (BIO-RAD).

4.3 Results

The chitosan membrane has a dense structure and a thickness of 5.5±0.23 μm (Fig.1a-b).

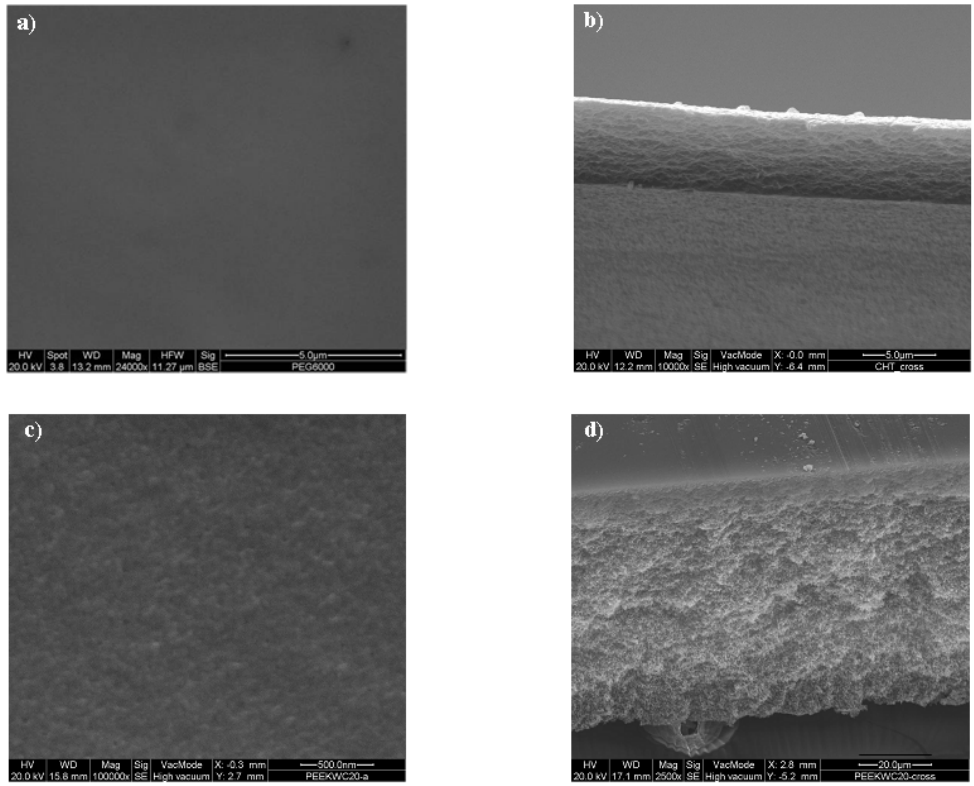

Figure 1

Figure 1: SEM's images of (a-b) chitosan and (c-d) PEEK-WC membranes; a) and c) membrane surfaces; b) and d) membrane cross section

Differently PEEK-WC membrane has an asymmetric structure with a gradient of porosity from one side to the other one (fig.1c-d). The membrane has a porous surface with a mean pore size of 55 nm and a thickness of 66±0.84 μm.

An IR spectrum of chitosan is shown in Fig. 2a.

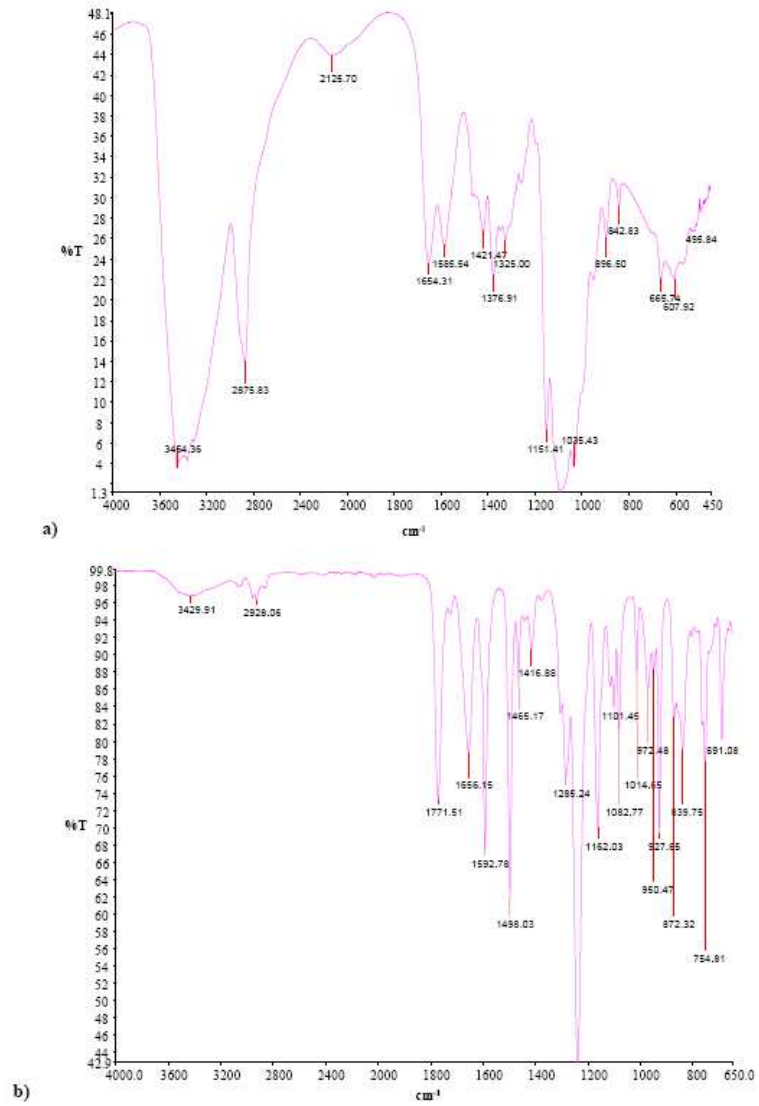

Figure 2: FITR spectra of a) chitosan and b) PEEK-WC membranes

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A sharp band at 3367 cm^{-1} has been attributed to -NH_2 and –OH group stretching vibration. Further, in the C-H stretch region of FTIR spectrum, the higher intensity peak at 2876 cm^{-1} is assigned to the symmetric modes of CH_2. In addition, the presence of one peak at 1420 cm^{-1} that was assigned to unsaturated carbon- carbon double bonds is observed in the spectrum. The peaks at 1654, 1585 and 1151 cm^{-1}could be assigned to strong N-H bending vibration of primary and secondary amide and C-O stretching vibration of ether linkage of chitosan backbone, respectively. From Fig.2b the typical chemical groups of PEEK-WC membranes were identified. A large band centred at 1768 cm^{-1} corresponding to superimposed ketonic and esteric C=O stretching modes, was observed in the PEEK-WC membrane spectrum.

Fig.3 shows the wettability properties of the chitosan and PEEK-WC membranes. Chitosan membrane surface displays hydrophilic character as demonstrated by time-related WCA and water sorption measurements (Fig3a):

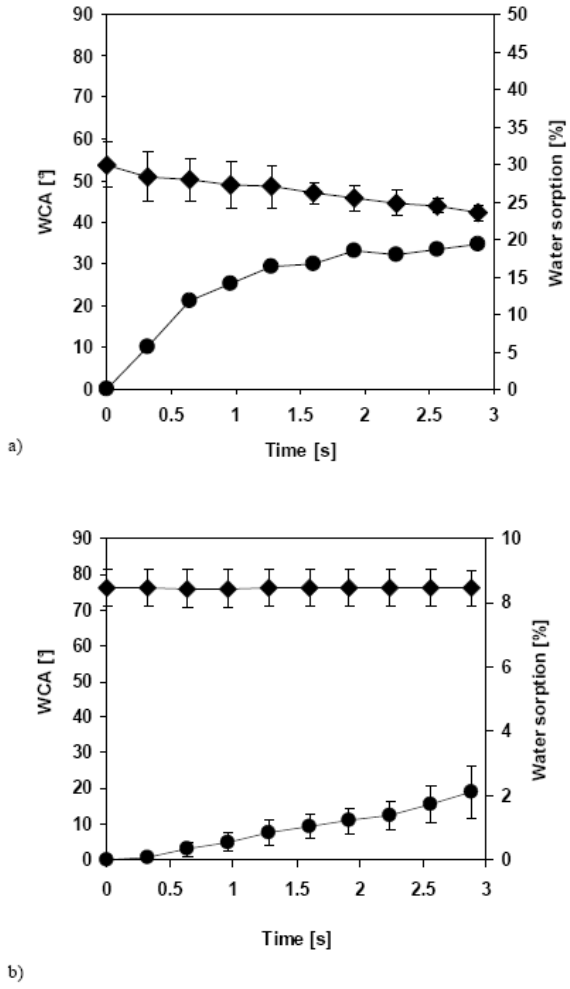

Figure 3: Time related water contact angle (◆) and water sorption (●) of a) chitosan and b) PEEK-WC membranes. The values are the mean of 10 measurements per sample ± standard deviation.

WCA decreased from 53.8±5.6° at t=0 to 42.17±1.8° at t=2.88 s and consistently the water sorption increased of 19%. Also the PEEK-WC membrane surface shows a hydrophilic character although the WCA value is higher (76±5.1°) with respect to the chitosan and remained constant with time (Fig.3b). Swelling and dissolution behaviour of chitosan membranes changed with time (Fig.4).

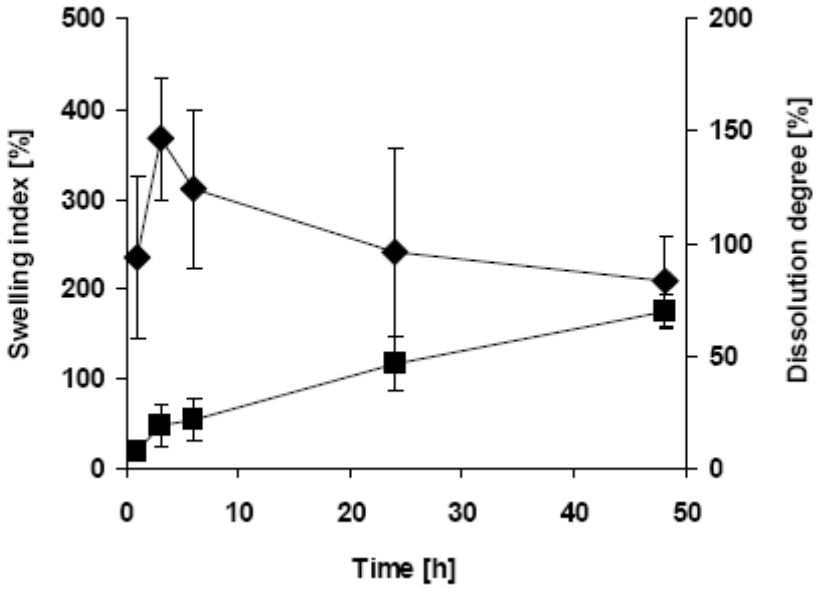

Figure 4: Swelling (◆) and dissolution (■) behaviour of chitosan membrane as a function of time. The values are the mean of 10 measurements per sample ± standard deviation.

The swelling index displayed a maximum at 3 h (about 366%) while the dissolution increased with increasing time reaching a value of 69% after 48 h.

As shown by SEM, cells easily adhered to the chitosan membranes, then proliferated during the first days of culture and spread to a confluent layer covering the membrane surface (Fig.5a).

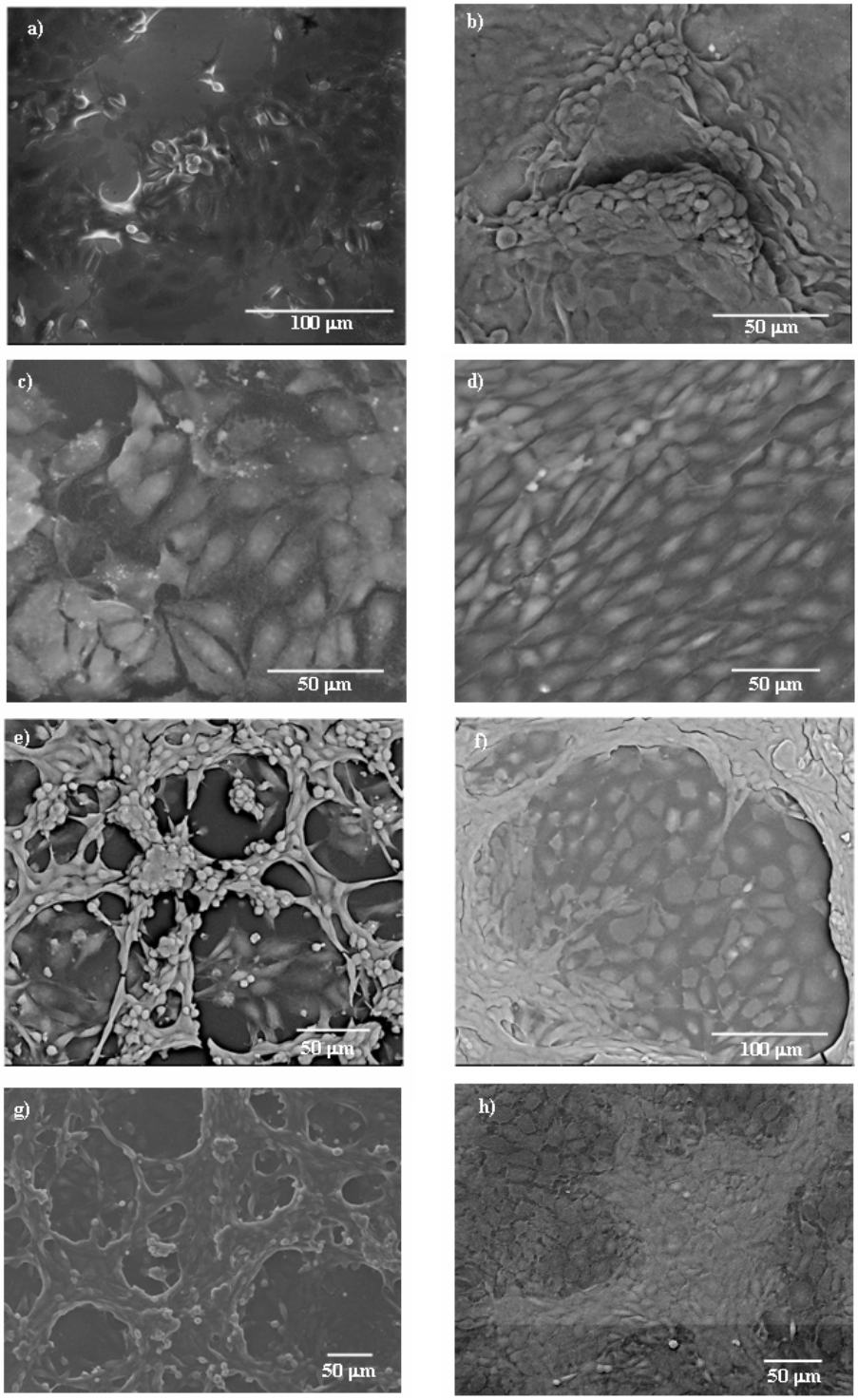

Figure 5: SEM images of rat embryonic liver cells after 7 days (a,c,e,g) and 16 days (b,d,f,h) of culture on: a-b) chitosan membrane; c-d) PEEK-WC membrane; e-f) collagen and g-h) PSCD at different magnification.

After saturation of the available space cells tend to form cord-like structures constituted of aggregates as result of cell proliferation (Fig.5b). On PEEK-WC membranes cells adhered and proliferated showing a polyhedral shape and a low aggregation degree with respect the chitosan membranes (Figs.5c-d). Cells exhibited a cell growth and tight cell-cell contact structure. A similar organization was displayed by cells cultured on collagen where the formation of cord-like structure was observed after 16 days of culture (Figs. 5f). As on the collagen, cells grown on traditional PSCD and formed similar two-dimensional layers (Figs. 5g-h). Further structural morphological investigations were performed with LCSM at 7 and 16 days of culture (Figs. 6).

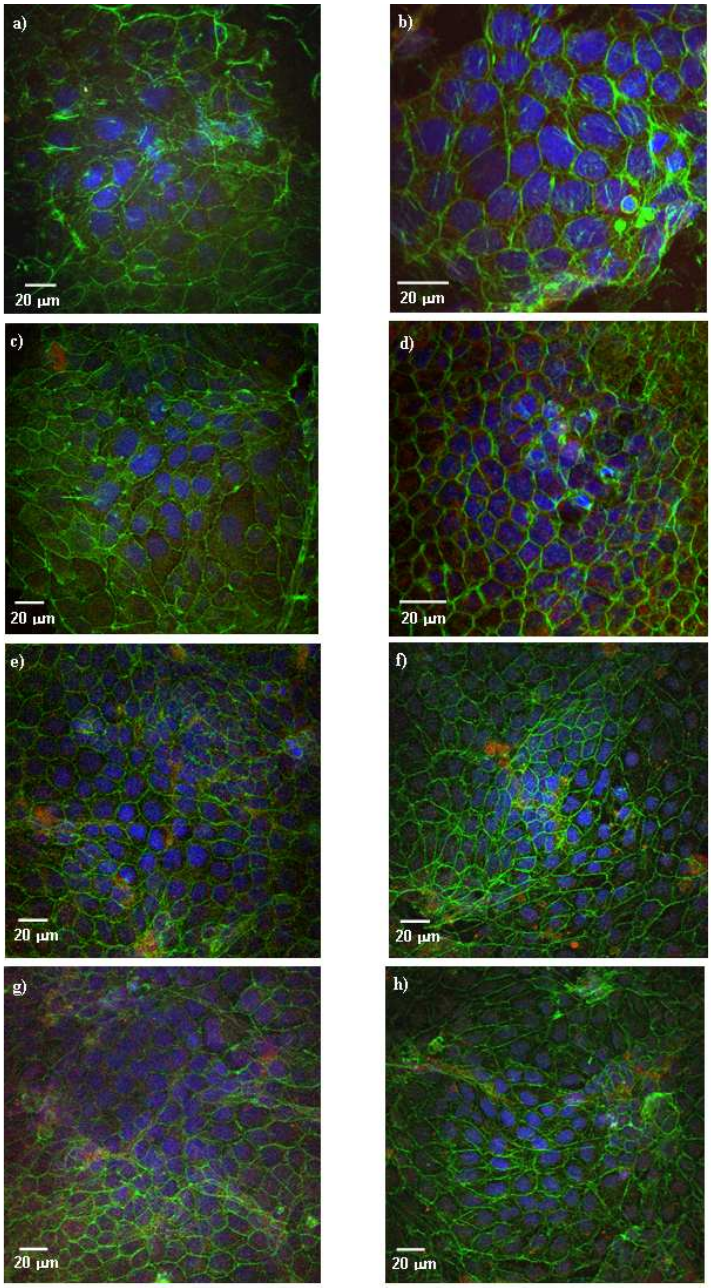

Figure 6: Confocal images of rat embryonic liver cells after 7 days (a,c,e,g) and 16 days (b,d,f,h) of culture on a-b) chitosan membrane; c-d) PEEK-WC membrane; e-f) collagen and g-h) PSCD. Cells were stained for actin (green), vinculin (red) and nuclei (blue).

The staining for actin and vinculin displayed the overall morphology of cells cultured on chitosan and PEEK-WC membranes, and the organization of the cytoskeleton proteins. After 7 days cells were found to typically have a distinct circumferential bundle of actin filaments immediately behind the free edges of lamellae while straight, thin bundles of filaments were observed in the central regions of the cells (Fig.6a). The presence of numerous focal adhesions observed under circumferential bundles of actin and at the ends of the straight bundles of actin filaments was detected. Cells on chitosan (Figs.6a) and PEEK-WC membranes (Figs.6c) appeared well spread, with typical polyhedral shape of parenchymal cells. After 16 days of culture cells maintained the organisation of actin fibres radially oriented in the area of cell-cell contact and the focal adhesion complexes (Figs.6b,d,f,h).

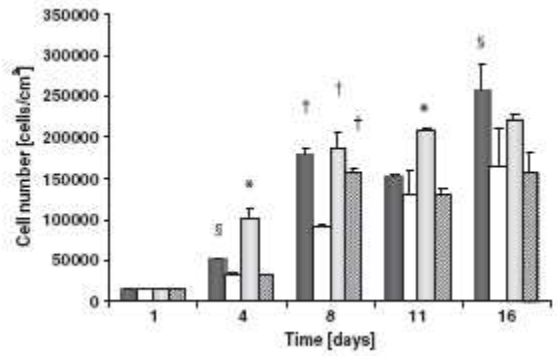

Figure 7: Proliferation of rat embryonic liver cells on chitosan membrane (full bar), PEEK-WC membrane (white bar), collagen (grey bar) and PSCD (dashed bar). The values are the mean of six experiments ± standard deviation. *p<0.05 vs all; §p<0.05 vs PEEK-WC and PSCD; †p<0.05 vs PEEK-WC.

Quantitative analysis of the cell proliferation is given in Fig. 7 that depicts the growth curves of the rat embryonic cells on the different substrates. The first observation of the cells occurs by day 4 in which about 5×10^4 and 10×10^4 cell/cm^2 are present on

chitosan membrane and on collagen respectively. By day 8 on collagen and chitosan membranes cell number is the same. On PEEK-WC membrane and PSCD the cultures reached saturation with values of 13×10^4 cell/cm^2 on day 11 whereas maximum counts on chitosan membrane and collagen reached on day 16. At this time cells approached 25×10^4 cell/cm^2 on chitosan membranes and 22×10^4 cell/cm^2 on collagen. After 16 days of culture the cells on the different substrates were slowly losing cells into the medium.

The expansion of cells in the long-term culture were paralleled by changes of the expression of specific proteins such as albumin as marker of mature hepatocytes, CK18 as marker mature liver cells and AFP as marker of hepatoblasts. Western blot at day 12 of culture demonstrated that besides the production of albumin and CK18 on the investigated substrates also the AFP was present in the culture medium (Fig.8).

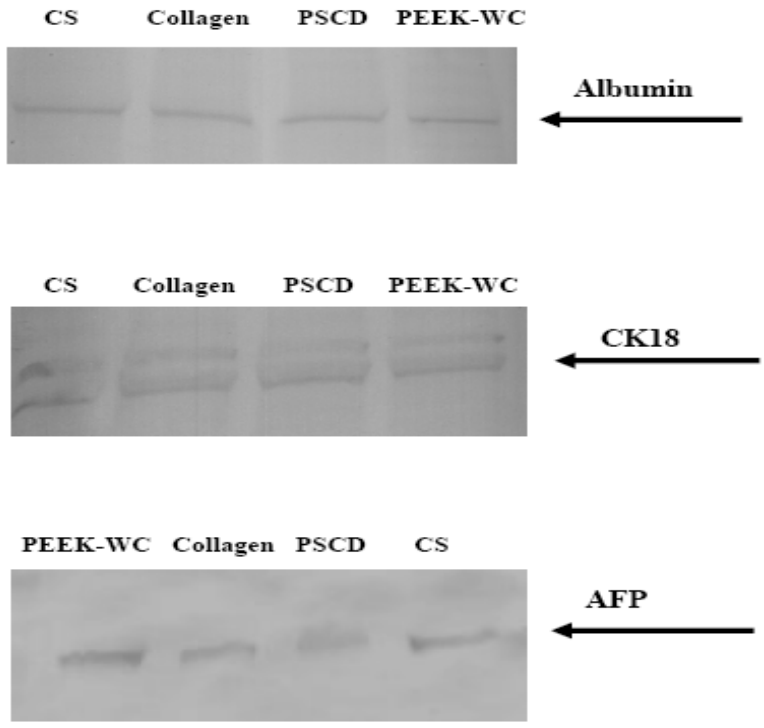

Figure 8: Western blot analysis of albumin, CK18 and AFP in the rat embryonic liver cells after 12 days of culture on the investigated substrates.

Interestingly cells exhibited during culture time a functional differentiation that led to the expression of liver specific functions such as albumin production and urea synthesis and drug biotransformation functions. During the first days of culture the albumin production increased reaching values of 0.543 ng/ml×day on chitosan membrane at day 6, then the secretion of albumin decreased at days 10-14 on all substrates (Fig.9a) although on chitosan membrane cells continued to synthesize albumin with higher rate (0.2 ng/ml×day) with respect to the other substrates.

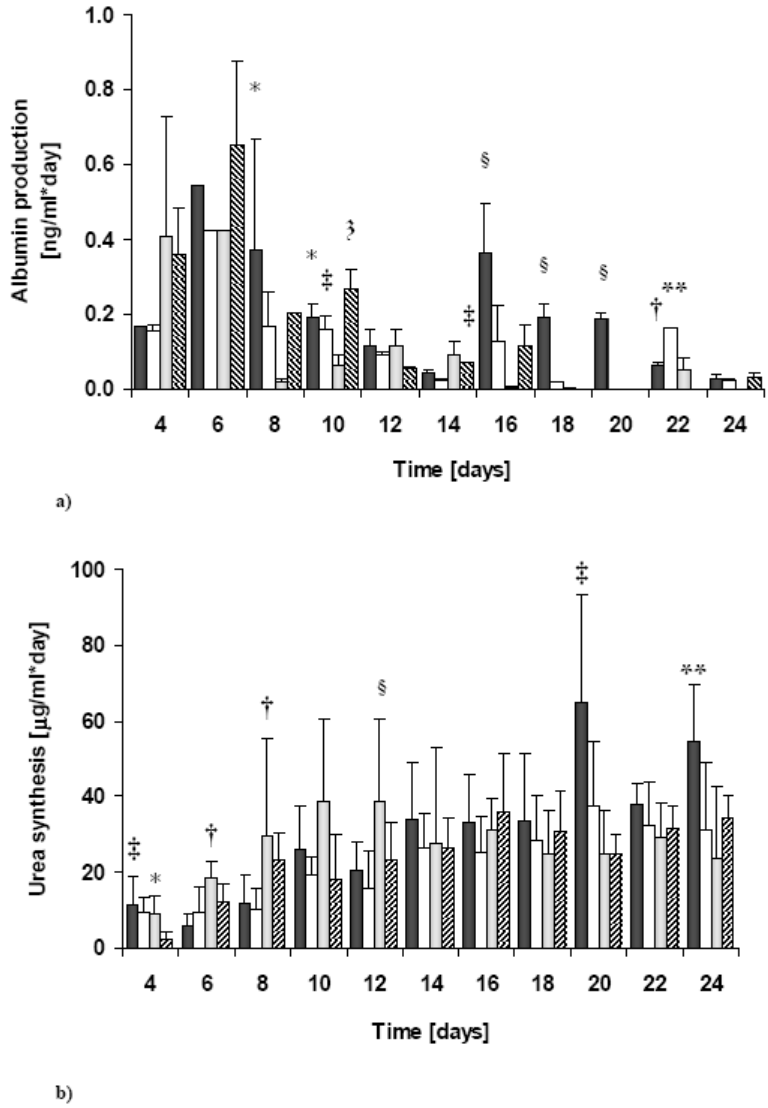

Figure 9: Liver specific functions of rat embryonic liver cells on chitosan membrane (full bar), PEEK-WC membrane (white bar), collagen (grey bar) and PSCD (dashed bar).

a) Albumin synthesis. The values are the mean of six experiments ± standard deviation. *p<0.05 vs collagen; §p<0.05 vs PEEK-WC, collagen and PSCD; †p<0.05 vs collagen and PSCD; ‡p<0.05 vs collagen; **p<0.05 vs chitosan, collagen and PSCD; zp<0.05 vs PEEK-WC and collagen.

b) Urea synthesis. The values are the mean of six experiments ± standard deviation. *p<0.05 vs CS, PEEK-WC and PSCD; ‡p<0.05 vs PSCD; †p<0.05 vsCS and PEEK-

WC; §p<0.05 vs PEEK-WC; **p<0.05 vs collagen and PSCD.

Urea was synthesised with rates that increased with time on all substrates (Fig.9b). Cells on collagen displayed higher levels of urea synthesis from day 6 to 10 and then remained stable whereas on chitosan membrane the synthesis of urea reached highest values on day 20. At this time urea was synthesised with a rate that is 6 fold higher with respect to the rate measured on day 4.

Biotransformation functions were tested by incubating cells with diazepam at the concentration of 10 μg/ml on day 7 and 16 of culture (Fig.10).

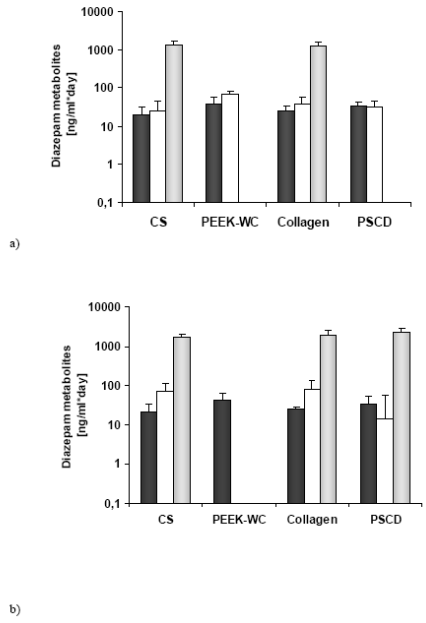

Figure 10: Diazepam metabolite formation of rat embryonic liver cells after 7 (a) and 16 days (b) of culture on membranes, collagen and PSCD. Oxazepam (full bar), nordiazepam (white bar), temazepam (grey bar). The values are the mean of six experiments ± standard deviation.

The diazepam metabolites of the phase I reactions that include oxazepam, nordiazepam and temazepam were detected. Differences were observed for the metabolite formation from embryonic liver cells cultured on different substrates.

On collagen the temazepam formation rate is higher with respect to the other metabolites both on day 7 (Fig.10a) and on day 16 (Fig.10b) with values of 1325.75 ng/ml×day and of 1895,50 ng/ml×day respectively.

Also the cells cultured on chitosan membrane displayed the ability of biotransforming the diazepam with the formation of all the metabolites involved in the pathways that lead to its clearance.

In particular the clearance of diazepam on chitosan membrane occurred with the formation of temazepam metabolite to a larger extent with respect to oxazepam and nordiazepam for both administrations, with a value of 1340.99 ng/ml×day on day 7 and a value of 1737.19 ng/ml×day on day 16.

The diazepam is completely metabolized also by the cells cultured on PSCD after 16 days whereas on PEEK-WC membrane only one metabolite was detected. From the first administration the complete pool of metabolites have been obtained only using the chitosan membrane as well as the natural substrate, collagen, suggesting the good performance of the chitosan membrane for the maintenance of functional integrity.

4.4 Discussion

A great deal of attention has been focused to the liver progenitor cells for the therapeutic potential and for their usefulness in the toxicological testing after their in vitro differentiation. The suitability of a matrix onto which cells can organize and develop in the proper environment is key for the differentiation and the maintenance of differentiated phenotype at the morphological and functional level. One of the critical steps in the development of membranes for liver regeneration is the design

and the selection of the biomaterial and the choose of chitosan as a polymer is related to its intrinsic properties of excellent biocompatibility and biodegradability.

Chitosan membrane may act as a synthetic extracellular matrix providing to the cells a structure with a well defined geometry and adequate physico-chemical properties. The degradation behaviour of the chitosan membrane plays a crucial role on the long-term performance of the tissue-engineered construct. We evaluated the swelling behaviour and the dissolution degree of the chitosan with time (Fig. 4). It can be easily seen that the swelling index value reached a maximum after 3 hours and then decreased slowly. Similar profile of swelling has also been reported from other authors [21-26] Since chitosan is hydrophilic and the diffusion of chitosan is faster, the films begin to swell prior to degradation. During, the initial stages of hydration process, bond cleaving and degradation of chitosan also occur, but the swelling surpasses the degradation [26]. The decrease in swelling can be attributed to due to the buffer salts, residual acetic acid etc. [25]. Furthermore when the swelling reached the maximum the continual degradation led to weight loss and the swelling index decreased to value of 209±50% at 48 hours while the dissolution degree reached the value of 69.7±7%.

It is well known that the morphology of cells is strongly affected by the intrinsic physico-chemical properties of the substrate where they are seeded. The adhesion of cells is related to quantitative expression of the membrane surface hydrophilicity, so the membrane surface can modulate directly the interaction with the cellular adhesion molecules and/or indirectly through the secretion of ECM proteins and consequently can elicit cell adhesion and functions [27]. This is consistent with our experimental results regarding the cellular morphological analysis, in fact the high wettability of both chitosan and PEEK-WC membranes allows promoting cell adhesion and proliferation on these substrates. As confirmed by laser confocal scanning observations on both membranes, cells kept their polygonal shape with a clear

reorganization in vitro of the cytoskeleton proteins for the entire culture period forming a parenchyma-like structure (Fig.6). It is interesting to note how cells proliferated on chitosan membrane to a large extent with respect to the other substrates. Such membrane offers to the cells a matrix that favour the initial step of cell adhesion due to the presence of protonated amino functions, at physiological pH, that interact with the anionic functions of the cellular membranes in the early-attachment phase until the cells synthesized the proteins of ECM that mediate the contact with integrins.

The functional differentiation of the rat embryonic liver cells on the chitosan and PEEK-WC membranes as well as on collagen and traditional culture dishes has been evaluated in terms of albumin production, urea synthesis and diazepam biotransformation. Differentiation of embryonic liver cells was traced-up by determining the expression for albumin, a typical marker for mature hepatocytes; AFP, a marker of hepatoblasts, which are bipotent cells giving rise to hepatocytes and bile-duct epithelial cells; CK18 a marker expressed by several liver cell types, and including bile ductal epithelial cells and hepatocytes [28]. The secretion of albumin, AFP and CK18, attested the differentiation into the hepatocyte cells [29]. To test whether the cells possess the liver specific functions we investigated the urea synthesis, albumin production and diazepam biotransformation functions during the culture time. It is interesting to note how cells maintain their liver specific functions for the whole culture time. The rate of urea synthesis increased with time whereas the synthesis of albumin was expressed at high levels at day 6 and then a decrease was observed concomitantly with the increased proliferation (Fig.9). This decrease can be explained considering the down regulation of albumin synthesis during proliferation process. The transcription rate of albumin gene is regulated by the interaction of a variety of promoter binding proteins, such as CCAAT/enhancer binding protein (C/EBP-α), C/EBP-β. Some literature studies suggested that the expression of C/EBP-α is inversely related to the proliferative state of the cells and that is

consistent with a role of the C/EBP-α as a factor that can function to maintain the quiescent state [30]. In our study we observed a decrease of albumin synthesis during the proliferation of hepatocytes that in part was recovered when the cell proliferation beginning to slow down.

Another important result concerns the biotransformation activity of cells (Fig.10). Diazepam is metabolised by liver cytochrome P450s to three major metabolites: N-desmethyldiazepam or nordiazepam, temazepam and oxazepam. The metabolic pathway of diazepam involves a variety of CYPs. Neville et al.(1993) reported that *p*-hydroxylation, 3-hydroxylation, and N-desmethylation are the pathways of diazepam metabolism in rat liver microsomes and that they were catalyzed by the respective P450 isoforms of CYP2D1, CYP3A2 and CYP2C11. [31,32]

The production of all three metabolites on chitosan membranes as well as in the collagen after 7 days of culture demonstrated the full specificity and activity of the phase I CYP isoenzymes achieved on these substrates while on PEEK-WC membranes and PSCD cells secreted only partially diazepam metabolites probably due to a partial expression to the isoenzymes responsible of the metabolic pathway. Cells continued to synthesize the three major metabolites of diazepam with culture time and after 14 days they shown a complete biotransformation of diazepam through the phase I reactions on chitosan membranes as well as on collagen an PSCD with a significant increase of the metabolic reaction rates.

4.4 Conclusions

In this study we investigated the expansion of rat embryonic liver cells and the expression of their differentiated functions on chitosan and PEEK-WC membranes prepared by phase inversion technique in comparison with collagen and polystyrene culture dishes (PSCD). Cells proliferated generating a significant increase in cell number and formed a structure close to liver parenchyma. Chitosan membrane provided optimal microenvironment conditions for embryonic liver cells, granting

them means to acquire and keep specific functions in a comparable way to those found in the collagen as reference system. Cells gained a functional differentiation showing the urea synthesis, albumin production and diazepam biotransformation at significant high levels particularly on chitosan membrane. On the basis of these results chitosan membrane is able to promote the expansion and functional differentiation of embryonic liver cells facilitating their use in liver tissue engineering and in pharmaceutical applications.

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Chapter V

Chitosan biodegradable films for neuronal tissue regeneration

6.1 Introduction

The design and the development of tissue-engineered products have benefited from many years of clinical utilization of wide range biodegradable polymers. Newly developed biodegradable polymers and novel modification of previously developed biodegradable polymers have enhanced the tool available to create clinically important tissue engineering applications. The increased demands placed on biomaterials for novel sophisticated medical implants such as tissue engineering constructs continues to fuel the interest in improving the performance of existing medical grade polymer and in developing new synthetic polymers.

Several biomaterials have been developing in tissue engineering application for support new tissue growth and repair; any biomaterial that is clinically used must have excellent properties in bulk as well on the surface [1]. Since it is the biomaterial surface that first comes into contact with the living tissue when the biomaterial is planted in the body, the initial response of the body to the biomaterial depends on its surface properties. Surface properties that can influence biocompatibility include surface charge, surface topography etc. [2] [3]. An effective approach for developing a clinically applicable biomaterial is to modify the surface of a material that already has excellent biofunctionality and bulk properties [4]. Widely used surface modification techniques include coating, oxidation by low-temperature plasma for better printing and adhesion. The physical properties of a polymer can also be altered by introducing a second polymer that improves the properties of the original polymer, such hidrophobility [5]. However, blending technologies with some additives may cause cytotoxicity; only acceptable additives can be used. In particular hippocampal neuronal cells for attach to substrate, need of extracellular matrix proteins, as

collagen, laminin, poly-D-lysine, poly-L-lysine, polilysine-alanin or fibronectin, etc [6]. For this reason many work are propose different surface modification for cell adhesion induce. Micropatterned substrate, whose surface was modified with electrodeposition of molecules such as polylysine, laminin, or both, is used for cell adhesion and neurite extension [7] [8]. Lysine-alanine polymer substrate has been demonstrated to enhance axon growth of neurons in a serum free medium in vitro [9]. Attachment and network formation of neurons were produced by contact printing of a peptide PA22-2 onto functionalised silicon oxide surface, in other work [10].

Grafted polyethylene glycol surfaces were used to create poly-D-lysine substrates permissive to cell attachment and growth, and neurite extension [11]; in other case embryonic hippocampal neurons are cultured on fluoropolymers modified with laminin-derived cell-adhesive peptides [12]. Poly ethylene-co-vinyl alcohol (EVAL) were modified by covalent bonding of lysine to improve cell behaviour in cultured neurons [13].

In this study i report on the preparation a chitosan surface for promote neuronal cells growth and neurites outgrowth. The ability to promote cell adhesion and neurites outgrowth was investigated in a modified chitosan (CS) surface and chitosan coated with poly-L-lysine (CS-Ply).

Chitosan is a biosynthetic polysaccharide that is deacylated derived of chitin. Chitin is a naturally occurring polysaccharide that can be extracted from crustacean exokeletons or generated via fungal fermentation processes. Chitosan is a b-1,4 linked polymer of 2-amino-2-deoxy-d-glucose; ti thus carries a positive charge from amine groups [14]. It is hypothesized that the major path for chitin and chitosan breakdown *in vivo* is through lysozyme, which acts slowly to depolymerise the polysaccharide. Chemical modification of chitosan produces materials with a variety of physical and mechanical properties. For example, chitosan film and fibers can be formed utilizing cross-linking chemistries and adapted techniques for altering from

other polysaccarides, such as treatment of amylase. Like hyaluronic, chitosan is a not antigenic and is a well-tolerated implanted material [1].

Neurons used in this experiment were isolated from hippocampus, which is a part of the forebrain, located in the medial temporal lobe. It belongs to the limbic system and plays major roles in learning, short term memory [15] and spatial navigation [16]. Hippocampal neurons, which are well-known for their plasticity and regeneration properties [17], are the best-characterised model for investigating polarization that occurs spontaneously during the first days of culture [18] [19].

The status of the cell growth on CS and CS-Ply surface was observed at different days of culture; neuron-surface interactions were analyzed over time in terms of size of cell body (soma), neurite outgrowth (legth) and viability. Metabolic behaviour of cells were compared between both surfaces; the ability of both substrates to reconstruction the neural network *in vitro* was also quantificated by measure of brain-derived neuronal factor (BDNF) secretion in these bio-hybrid systems. This neurotrophic factor is product from hippocampal cells in the sinapsys connections, during the development. BDNF support the survival of existing neurons and encourage the growth and differentiation of new neurons and synapses [20].

Mice born without the ability to make BDNF, suffer developmental defects in the brain and sensory nervous system, and usually die soon after birth, suggesting that BDNF plays an important role in normal neural development [21].

The biocompatibility of CS surface with hippocampal neuronal cells could be used in future for some biomedical application as neurodegenerative disease and for drug testing.

6.2 Materials and Methods

6.2.1 Membranes preparation

Membranes were prepared by inverse phase techniques using the direct immersion precipitation method [22]. CS membranes can be obtained by casting 4% chitosan solutions and 1% of PEG 6000 in acetic acid at 2 % w/v concentration at room temperature. Chitosan was dissolved in 2 % acetic acid solution and then PEG was added to the chitosan solution. The mixing solution was cast and dried a room temperature; after drying the membrane was immersed in a solution of 1 % NaOH. Finally, the membrane was washed in deionized distilled water and dried for test [23].

Some membranes were modified by coating with poly-L-lysine (PLL) (MW 30000-70000), dissolved in a boric acid/sodium tetraborate solution (1:1) to a final concentration of 0.1 mg/mL, in order to have the same functional groups over the surfaces with a density of 40 $\mu g/cm^2$ [24]. The membranes were coated with poly-L-lysine in PBS and incubated for 3h and then the excess of poly-L-lysine (PLL) solution was removed and dried. Poly-L-lysine–coated Polystyrene culture dishes (PSCD) were used as a control.

6.2.2 Membrane physiochemical properties

The surface morphologies of CS membranes were studied using a scanning electron microscope (SEM) (Quanta 200F ESEM, FEI, USA). All samples were dried in vacuum at room temperature. From photographs obtained, it's possible to observe the typical morphology of the membrane, as surface and cross section.

The wettability of the membranes was characterized by means of water contact angle (WCA) measurements. Contact angle of water droplets were measured (sessile drop) at room temperature with a CAM 200 contact angle meter (KSV Instruments LTD, Helsinki, Finland). The sessile drop was formed depositing water onto the membrane surface with an automatic microsyringe. WCA measurements as a function of the contact time were performed on all surfaces. At least 30 measurements on different

regions of each sample were averaged per each CA value. Standard deviations are indicated as error bars.

The water sorption of the membrane surfaces during the time was evaluated by following the decrease of drop volume in contact with the surface using the relation:

$$\text{surface water sorption (\%)} = \frac{\text{drop volume}_{(t=0)} - \text{drop volume}_{(t=i)}}{\text{drop volume}_{(t=0)}} * 100$$

6.2.3 Cell isolation and culture

The hippocampus of both hemispheres was dissected from the brain of postnatal days 1-3 (PND1-3) hamsters (*Mesocricetus auratus)*, removed and collected in falcon tubes in Neurobasal medium A (Invitrogen Corporation, Milan, Italy) containing 0.02% BSA (Sigma, Milan, Italy). The tissue was digested in a Neurobasal medium A containing 0.1% papain (Sigma) and 0.02% BSA (Sigma) for 20 min at 37°C [25]. Ten minutes after digestion, the tube containing the tissue was mixed and at the end of digestion, the supernatant containing papain was removed and Neurobasal medium A supplemented with B27 (2% v/v; Invitrogen Corporation, Milan, Italy) penicillin-streptomycin (100 U/mL), glutamine 0.5 mM (Biochrom AG), 5ng/mL basic fibroblast growth factor (b-FGF; Sigma) was added to the remaining pellet. Samples were gently triturated mechanical by using a sterile Pasteur pipette with a wide opening to dissociate larger aggregates. After sedimentation of the aggregates the supernatant was removed and transferred into tubes containing 1% papain inhibitor in Neurobasal medium A and 1% BSA, as described elsewhere [26]. The samples were centrifuged at 1300 rpm for 10 min at room temperature and cell pellets were gently re-suspended in Neurobasal medium A containing B27 supplement, penicillin-streptomycin, 0.5 mM glutamine, 5ng/mL b-FGF. Serum-free B27 supplemented Neurobasal medium A seems to have a beneficial effect on the growth and differentiation of hippocampal neurons, as suggested by other researchers [27, 28]. The viability of the cells after this isolation procedure was assessed by trypan blue

test and resulted to be 97±2%. Cells were seeded on the different membrane surfaces at $2.5x10^5$ cell/cm^2 density. Controls without cells were prepared for each kind of substrate. Cells and controls were incubated at 37°C in an atmosphere containing 5% CO_2. Cultures were fed every 4 days replacing half of the medium at each feeding.

6.2.4 Immunofluorescence of neuronal cell cultures

The morphological behaviour of neurons cultured on the different membranes were investigated and compared to PSCD as controls. Representative images of 4h, and 16 culture days displaying the distribution of the neuronal cytoskeletric marker, βIII-tubulin, and axon marker, growth-associated protein-43 (GAP-43) that were observed *in vitro* by a LCSM (Fluoview FV300, Olympus, Milan, Italy). 6 samples for each substrate were analyzed.

The immunofluorescence method specific for hippocampal cell cultures was conducted by using primary antị-βIII-tubulin followed by secondary antibody conjugated with FITC fluorochrome on samples previously fixed and permeabilized [21,29]. Specifically, the neuronal cells were fixed in paraformaldehyde (4%) for 15 min. Fixed cells were permeabilized with 0.25% Triton X-100 in PBS for 10 min and subsequently blocked with 1% BSA for 30 min at room temperature. The cultures were then rinsed three times with PBS and incubated with the monoclonal antibodies antị-βIII-tubulin (1:100; Sigma, Milan, Italy) and anti-GAP-43 (1:100; Sigma, Milan, Italy) overnight at 4°C. Afterwards, neuronal cells were rinsed with PBS and incubated with fluorescently labelled secondary antibodies FITC-conjugated (1:100; Invitrogen) and TRICT-conjugated (1:100; Invitrogen) in PBS for 60 min at room temperature. The cells were counterstained with DAPI (200 ng/mL; Sigma, Milan Italy), mounted by using a fluorescent mounting medium and observed at LCSM [26, 29].

6.2.5 Neuronal morphology CS and CS-Ply membranes

The immunofluorescence samples displaying the distribution of βIII-tubulin and GAP-43 in hippocampal cells grown on the different membranes were used in order to analyze *in vitro* some morphological parameters such as the area filled by neurons and axonal length especially at 4 and 16 days, periods in which the different neuronal elements and synaptic complexes, respectively, are fully formed. Quantitative evaluations of these morphological parameters were determined for distinct cell fields (100μm×100μm) at the two different developmental stages. NIH-Scion Image software was used in order to quantify the area filled by neurons, expressed as percentage of the total membrane area, and the axonal length (μm ± s.e.m.).

6.2.6 Sample preparation for SEM

Samples of cell cultures were prepared for scanning electron microscopy (SEM) (Quanta 200F ESM, FEI, USA) by fixation in 2.5% glutaraldehyde, pH 7.4 phosphate buffer, followed by post-fixation in 1% osmium tetroxide and by progressive dehydration in ethanol. Samples were examined at SEM and representative images displaying both neuronal structural features and adhesive properties on the different membrane surfaces were obtained at 8 days *in vitro.*

6.2.7 Biochemical assays

Neuronal medium was collected from the different cell culture membranes in pre-chilled tubes at critical developmental *in vitro* stages and stored at -80°C until assays.

The glucose concentration in the medium was detected by using Accu-Chek Active (Roche Diagnostics, Monza Italy). To assay the neuronal brain derived neurothrophic factor (BDNF) secretion, a sensitive BDNF ELISAs immunoassay (Promega Corporation WI, USA) was carried out on samples collected from 6 different isolations. BDNF Elisa was performed as follows: ELISA plates were coated with 100 μL of anti-BDNF monoclonal antibody overnight at 4°C. After washing, 100 μL

of cell culture supernatant was added to the wells and left for 2 hours at room temperature. Thereafter the wells were washed five times and incubated with 100 µL of anti-human BDNF for 2 hours at room temperature. After washing five times the wells were covered for 1 hour with anti IgY horseradish peroxidase conjugate and then 100 µl of Tetramethylbenzidine were added for 10 min. The reaction was blocked with 100 µl of 1N HCl and absorbance was measured at 450 nm using a Multiskan Ex (Thermo Lab Systems).

The statistical significance of the experimental results was established according to the ANOVA test followed by Bonferroni *t*-test ($p < 0.05$).

6.3 Results

The quality of the membrane was evaluated by observation of SEM images. CS membranes are characterized by a dense structure with microfracture, which confer rougher profile to the surfaces (Fig.1a). The cross section shows a homogeneous distribution of polymers in all thickness, without skin layer.

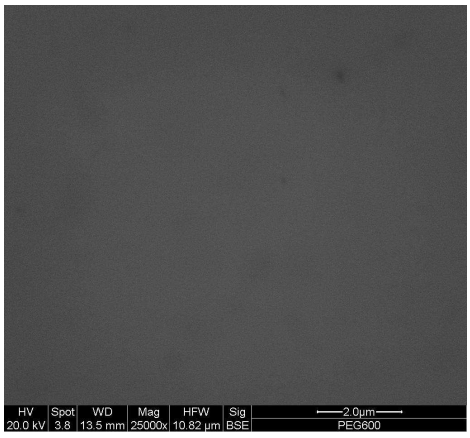


Figure 1 Scanning electron micrographs of CS surface membrane.

In order to have more information about the physico-chemical properties of the membranes we investigated the time-related water contact angle and water sorption.

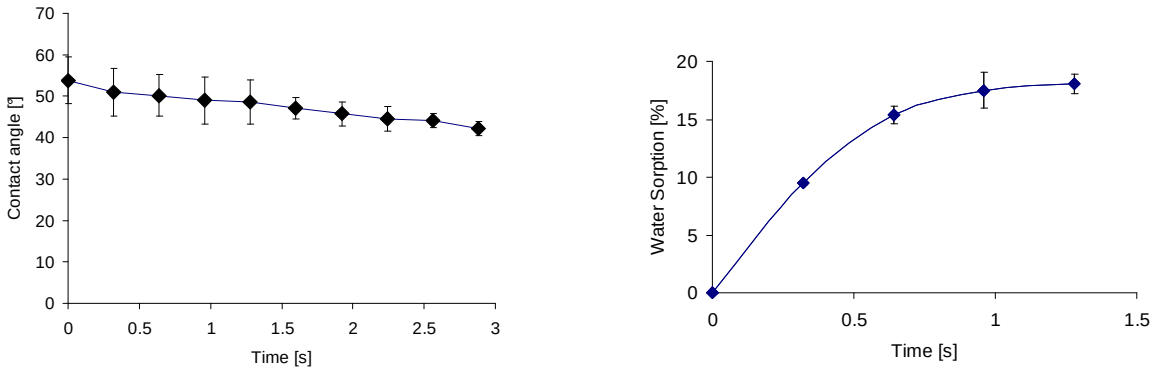


Figure 2: a) Time-related water contact angles measured on chitosan membrane surface. The reported values are the mean of 30 measurements of different droplets on different surface regions of each sample ± standard deviation. b) Surface water sorption percentage of chitosan membrane. The reported values are the mean of 30 measurements of different droplets on different surface regions of each sample ± standard deviation.

Fig. 2 shows static water contact angle values for native native membranes measured at t=0 when the water droplet contacts the membrane surface. Native chitosan membranes have a hydrophilic surface character in fact the water contact angle measured on this membrane was 53.8° ± 5,6° at t=0 s. The water droplet was absorbed in 1, 2 seconds.

After characterization of morphological and physiochemical properties, CS and CS-Ply membranes were used for cell culture experiments.

Primary hippocampal neuronal cells obtained from 1, 2 days year's old *mesocricetus auratus*, can be plated at low density on CS and CS-Ply. By observation of sequential

photography, it's possible to identify characteristic morphological changes that occur in the development of hippocampal neurons in culture.

Substrates have characteristics of transparency therefore permitted the online observation of the cells with time by an inverse light microscope. In fig. 3a, 4a, after 4h of culture, spherical cells attached to the CS and CS-Ply and from some sites "minor processes" develop. At 4 days of culture, polarity first, becomes evident, cells appear polarized, and cell's processes will become the axon and dendrites . Axons and dendrites continue to grow at rapid rate, but the remaining minor processes undergo little net elongation. A small number of nonneuronal cells also are present.

With subsequent development, the density of the axonal network increases, dendritic arbors become more elaborate and highly branched, synaptic contacts develop in a large numbers, dendritic spines appear, and spontaneous electrical activity propagates throughout the neuronal network . Cells increase the size of cell body and the diameter of axon and dendritic processes. A complex neuronal network was observed after 16 days of culture. The neuronal maturation is highly dependent on cell interactions with the substrate; pictures demonstrate CS and CS-Ply membrane premise the neuronal development (Fig.3).

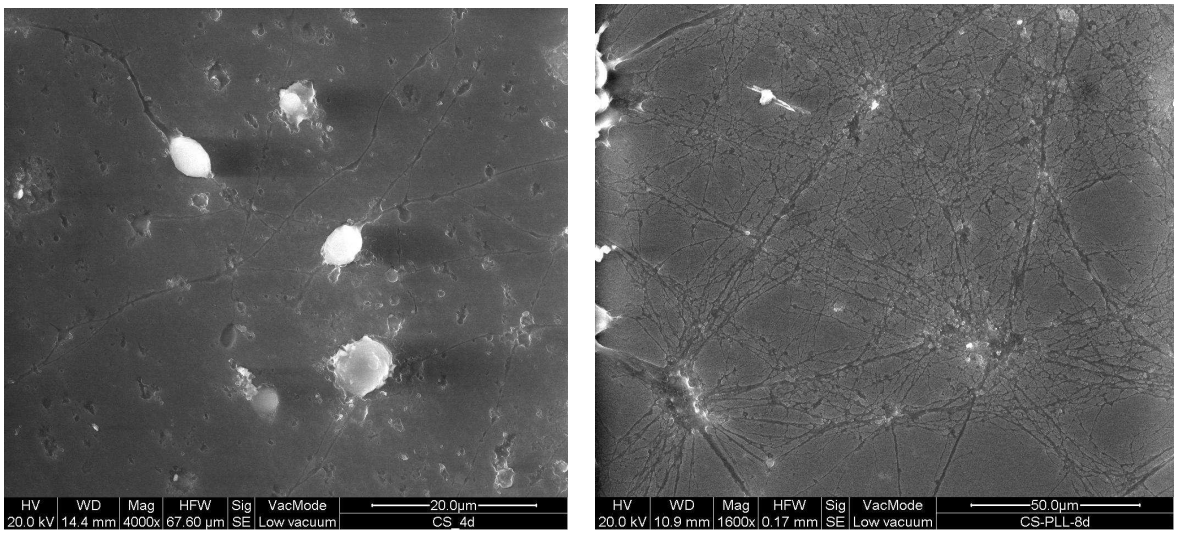


Figure 3: SEM's images of hippocampal neurons on: a) CS after 4 days of culture and b) CS-Ply after 8 days of culture**.**

Axons frequently course along the dendrites and, by light microscopy, such axons cannot be distinguished from the underling dendrites. These features can be resolved by intracellular injection of fluorescent dye such as immunocytochemical markers. The presence of the machinery for protein synthesis in dendrites, but not in axons, offers an alternative and complementary method for distinguishing dendrites from axons and assessing their differentiation.

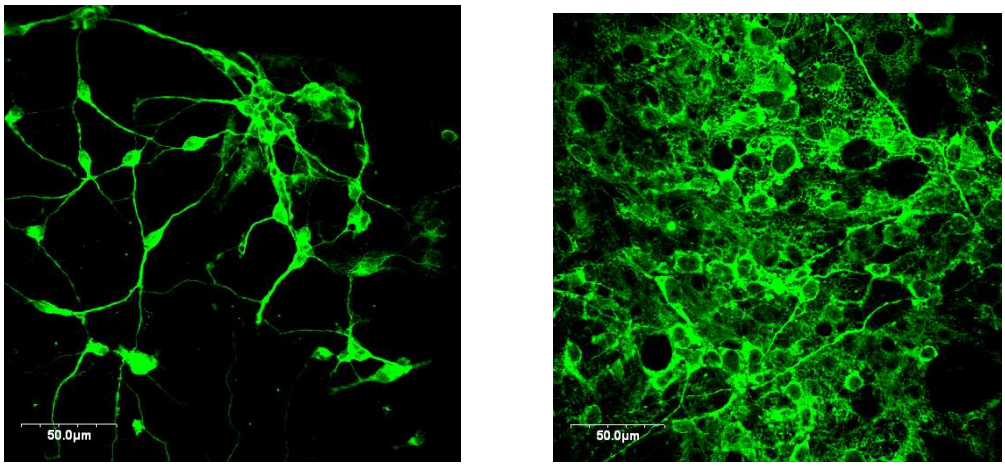


Figure 4: Confocal laser micrographs of hippocampal neurons traced for entire culture period and from representative images after16 days of culture it was possible to evaluate the distribution of βIII-tubulin (green) and the axonal marker GAP-43 (red) on: CS membranes. Cell nuclei were labeled with DAPI (blue)

The confocal images of the neurons, showed the localization of βIII-tubulin (green) in the neuronal network (Fig.4). This cytoskeletal protein was present in the soma and in all neuronal processes, while the axonal growth cones were visualized through the localization of GAP-43 (red), a specific protein involved in the regulation of axonal outgrowth.

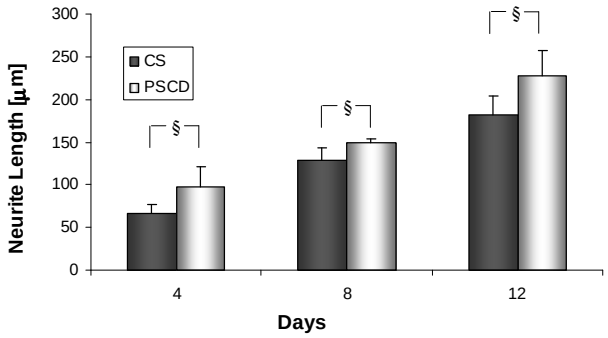


Figure 5: Analysis of morphological parameters of hippocampal cells on CS, CS-Ply and PSCD surfaces:

a) Area soma of hippocampal neurons after 4, 8, 12 and 16 days of culture on CS and CS-

Ply surfaces. Data were expressed as μm ± s.e.m. and evaluated according to

b) Axonal length of hippocampal neurons after 4, 8, 12 and 16 days of culture on CS and CS-Ply surfaces. Data were expressed as μm ± std.dev. and evaluated according to *t* –test. §p<0.0001.

To emphasize this important result, the morphology of cells was analyzed, by measure of size of cellular body and neurites outgrowth (Fig.5). The soma area is smaller in CS and CS-Ply respect to PSCD, demonstrating the absence of cell spreading (5a). Also neuronal network developed above the membrane surface at similar levels to PSCD and the cells exhibited a good neurite outgrowth (5b). Quantitative analysis confirmed that hippocampal axons developed on the other two substrates similar to PSCD culture.

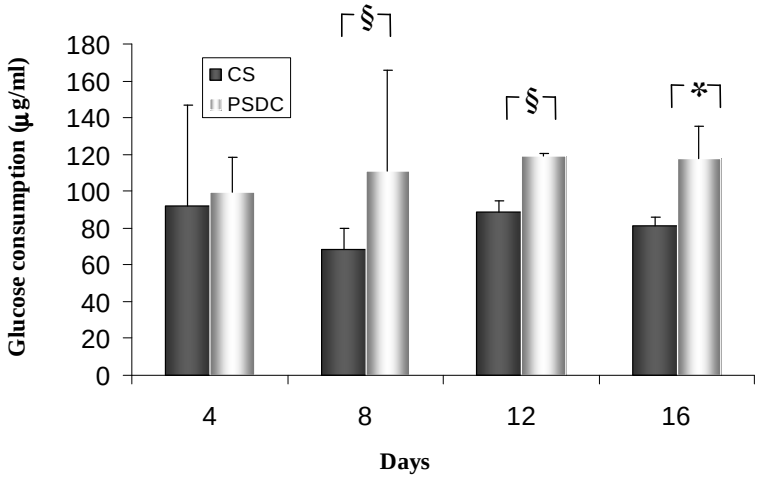
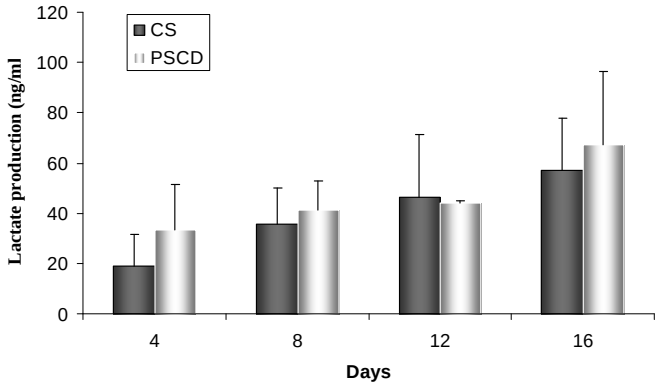


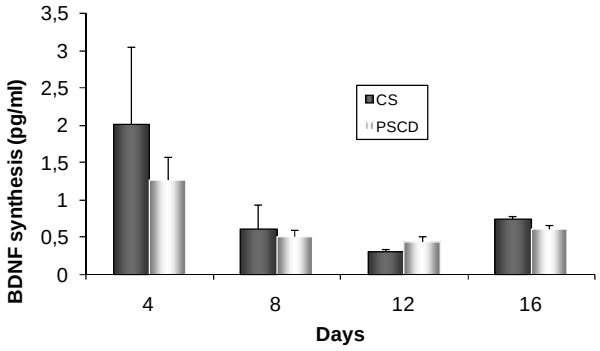
Figure 6: Metabolic activity of hippocampal neurons cultured on the different

membranes.

Glucose consumption of hippocampal neurons on the different membranes on day 4 (black bar), day 8 (white bar) and day 16 (grey bar) of culture. The values expressed as μg/mL ± std.dev. are the mean of 6 experiments and evaluated according to *t*-test. * $p<0.05$; § $p<0.001$.



a) Lactate production of hippocampal neurons on the different membranes on day 4, 8 day a day 12 of culture. The values expressed as μg/mL ± std. dev. are the mean of 6 experiments.



b) BDNF secretion of hippocampal neurons on the different membranes on day 4 (full bar), day 8 (empty bar) and day 16 (grey bar) of culture. Data were expressed as pg/mL ± std. dev. are the mean of 6 experiments.

There aren't statistically significant differences on the glucose consumption between CS, CS-Ply to respect PSCD. The glucose consumption remains constant throughout the culture period (Fig. 6a). The lactate production rate was lower on CS and CS-Ply then PSCD throughout the culture period, suggesting a much better oxygenation of the cells on both membranes.

The BDNF secretions is higher on CS membranes than PSCD, this higher levels of BDNF is maintained for 16 days of in vitro culture (Fig. 6d). The evaluation of the metabolic activity showed that cells maintained their functions at high levels throughout the entire culture period. This demonstrates CS and CS-Ply support cell viability and functions.

6.4 Discussion

This study demonstrates the successful adhesion and growth of primary neurons isolated from the hippocampus on CS membranes. CS Membrane was prepared with phase inverse technique and characterized with SEM for evaluation of membrane surface. At SEM's analysis CS membranes appeared with dense and homogeneous structure, particularly interesting for hippocampal cells culture [1]. CS membrane displayed a wettable membrane surface (Fig.2).

CS membrane bio-hybrid system promotes cell response and cell interactions without poly-L-lysine Many biomaterials, for promote the adhesion and modulate the growth, are coating with different extracellular matrix. Adsorbing protein encourage cell attachment [30-31] with distribution of positively charged surface [32]. In this study its possible demonstrates the important role of CS in the long-term hippocampal cells culture in absence of permissive adhesion's protein.

The cell biocompatibility of CS was tested by analysing adhesion and neurite extension of neurons in culture. Neurons are unpolarised cells after isolation they polarized and establish axons and dendrites during the first days of culture both on

CS and CS-Ply membrane (Figs. 3-4). The density of axonal network increases with the time, and neurites become more elaborate and highly branched on the both substrates.

Neuronal cells on both membrane, displayed the typical morphology of hippocampal neurons that included a primary apical axon and several dendrites with multiple ramifications, developed from cell body (soma). On CS membranes neurons have shown a smaller soma than PLL while the ramification of their extending processes is similar. There were no statistically significant differences between CS, CS-Ply membrane and PSCD in the neurites length (Figs.7a, b).This results demonstrate that Cs and CS-Ply membranes seem to be more supportive of neurite outgrowth modulating the development process of the neurons. Neurons have been shown to adhere, and growth on polymer by surface modification, with proteins such as fibronectin a vitronectin [33]. Other Authors found Poly(D,L-lactic acid) macroporous guidance scaffolds coated with laminin (foams) for neuronal survival, axonal regeneration were studied [34].

This study shows that hippocampal cells can growth and differentiate, extending neurites, on biocompatible surfaces without protein coating.

The correct and stable hippocampal neuronal formation is supported by the evaluation of some major cytoskeletal markers such as βIII-tubulin and GAP-43. Indeed, the constantly intense distribution of βIII-tubulin, specific for neuronal processes and soma, up to 16 days of culture, structured neuronal networks.

The metabolic functions of neurons on membranes were expressed at high levels demonstrated that, cells adhered on the membranes are functionally active for 16 days of culture (Fig. 8). The glucose uptake is similar on CS and PSCD culture, while lactate production on CS membrane is less to PSCD, demonstrated that the cells adhering on Petri dishes consume nutrients in a less grade of oxygenated medium to

the CS membranes. The results demonstrated that the CS membrane improves the oxygen uptake better than PSCD culture.

BDNF acts on certain neurons of the central nervous system and the peripheral nervous system, helping to support the survival of existing neurons and encourage the growth and differentiation of new neurons and synapses [35]. In the brain, it is active in the hippocampus, cortex, and basal forebrain—areas vital to learning, memory, and higher thinking [36]. BDNF itself is important for long-term memory [37]. BDNF was the second neurotrophic factor to be characterized after nerve growth factor (NGF).Various studies have shown possible links between low levels of BDNF and conditions such as depression, schizophrenia, Obsessive-compulsive disorder, Alzheimer's disease.

The high levels of BDNF on CS and CS-Ply membrane confirm the viable status of hippocampal neurons throughout the culture period. Hippocampal neurons exhibited higher BDNF's secretion on CS membranes than PSCD; This demonstrate that cells on CS membrane have a maturation stage faster than hippocampal cells on PSCD, developed highly branched neurites and a more complex network.

6.5 Conclusions

These results showed that Chitosan promotes survival and neurite outgrowth of hippocampal cells in vitro. Neurons in culture on CS membrane are vitals for 16 days and they develop neurite outgrowth in absence of proteins that promote of cell attachment and growth. The unaltered cytoskeletal structure of soma and development processes is confirmed by βIII-tubulin marker. The elevated secretion of BDNF show specific metabolic functions of maturated hippocampal cells in culture. Chitosan gels, powders, films and fibers have been formed and tested for applications such as membrane barriers in dynamic cell culture for regenerate neural tissue in a

well-controlled microenvironment. Chitosan membrane could be used for prepare membranes and matrices suitable for several tissue engineering applications.

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Chapter VI

Biodegradable microstructured membranes as in vitro model of peripheral nerves

6.1 Introduction

The number of recently developed functional scaffolds for tissue engineering and likewise peripheral nerve regeneration is large. Until now the ideal physico-chemical compositions of such materials and the ideal surface structure and functionalisation have not yet been found. Although peripheral nerves show capacity for regeneration after injury, the regeneration depends on the type of the gap and the length of the gap. Nerve regeneration competes with formation of scar tissue, without appropriate interventions, and results in a permanent loss of peripheral nerve regeneration. The microstructure and architecture of the scaffold together with the surface chemistry exert profound effects on cell distribution, morphology and alignment and, importantly, cell proliferation and differentiation, which underpin correct tissue development [1-3]

The aim of the study was to evaluate and characterise a novel biodegradable membrane of poly urethane (PU) and polycapro-lactone (PCL) for peripheral nerve regeneration. Was investigated the membrane structured surface which could enhance cell growth. The membrane-cell interactions were studied using model cell lines (SH-SY5Y).

In most of their current applications, articles fabricated from polyurethanes show excellent long term stability in their mechanical properties and resistance to chemical attack. The initial mechanical properties of PUs are also well suited for their usage in a number of bio-medical devices. The chemical composition of commercially available and experimental PUs varies widely and this range of chemical structures can be found both in the soft segment, which usually has a polyether or polyester structure, as well as in the hard segment, which is composed of a chain extender and a diisocyanate. PUs intended for biomedical applications, however, are structurally far less diverse and are commonly based on polyether soft segments [4].

It is noteworthy that poly (ether urethanes) implants have survived as a commercial products for decades [5]. Lastly, literature reports have suggested modifying poly(ether)urethanes (PU) in an

attempt to improve their properties for tissue engineering applications [6]. PU is hydrolytically stable; however, the presence of ether linkages in PU soft segments makes them susceptible to chemical degradation [7,8]. Biodegradation in poly (ether-urethanes) was observed as severe localized embrittlement of the surface. The chemical changes associated with biodegradation were observed as localized oxidation of the soft segment and hydrolysis of the urethane bonds joining hard and soft segments [9]. The participation of molecular oxygen in the degradation mechanism is supported by studies which showed that poly (ether-urethane) degradation *in vitro* correlated with oxygen diffusion into polymer bulk after surface oxidation [10]. Model studies have found that PUs can be degraded oxidatively [10,11], for instance by hydrogen peroxide. The polyether segment is thought to be the structural element that is most susceptible to oxidative breakdown while it is highly resistant to hydrolysis. The details of oxidative attack in the biomedical environment have not yet been elucidated, but it appears reasonable to surmise that the mechanism of oxidative degradation of the polyether segment involves radical chain reactions similar to those which degrade other polymers such as polyolefins, and chain cleavage. Under normal atmospheric conditions, PUs are relatively stable towards oxidation; however, metabolic products present in biomedical situations, such as peroxides secreted from macrophages can have sufficient oxidative strength greatly to enhance the degradation reactions [4]. Poly(ε-caprolactone) (PCL) is a polymer demonstrating such long degradation rates. However, the mechanical strength of PCL is inherently poor [12]. In our lab, we have developed methods to make PCL films with polyurethane, and thus, improve the mechanical properties and at the same time the biodegradation of components that promote the peripheral nerve regeneration.

In this study we have investigated the ability of PCL/PU and Chitosan (CS) membranes to promote the expansion of SHSY5Y cells, like a peripheral nerve *in vitro* model, utilizing traditional substrates such as PolyStyrene Culture Dishes (PSCD) as references.

6.2 Materials and Methods

6.2.1 Membrane preparation

The PCL-PU membranes were prepared by phase inversion technique. The PU (Pellethane 2363-80°, Dow Chemical Noderland BV, Deefzijl, Netherland) was dissolved at 15% (wt/wt) while the PCL (Sigma-Aldrich) at 28%, both in formic acid (99%) and stirred until they became clear. Two solutions were mixed together at ratio 1:2 in order to reach the final concentration of solvent around

80%. The solution was cast on glass plate by means of commercial knife (Elcometer, Adjustable Bird Appl 0-250 μm) and dried at room temperature. The membrane was immersed in a solution of 1% NaOH after drying. Finally, the membrane was washed in deionised distilled water for the test.

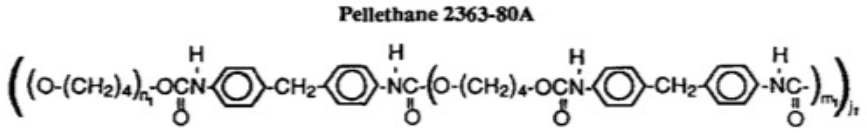


FIG. 1 STRUCTURE OF IMPLANTABLE POLY(ETHER URETHANE).

6.2.2 Membrane characterisation

The membranes were characterised by Fourier Transformed Infrared Radiation (FT-IR) analysis in order to identify the specific chemical groups of the polymers. The spectra were recorded on a Perkin Elmer 1300 spectrometer.

Differential scanning analysis (DSC) measurements were carried out on DSC2010 (TA Instruments). The DSC curves were recorded under a nitrogen atmosphere by setting a heating rate at 10°C /min.

The wettability properties of all membranes were characterised by using the water contact angle measurements by sessile drop methods and water sorption at ambient temperature by using CAM 200 contact angle meter (KSV Instruments LTD, Helsinki, Finland). Results are the mean of ten measurements of different regions of the sample surface. All measurements were repeated for six times.

Swelling and dissolution tests were performed for PCL/PU membranes. Membrane samples (1 mm×1 mm) were weighted and then put in 1 ml phosphate buffered saline (PBS) at 37°C. Swollen membranes were drawn at various times and were weighted again. The swelling index (SI) was calculated as: $\%SI = \frac{Ws - Wi}{Wi} \times 100$ where Wi and Ws are the sample weights before and after incubation in PBS respectively. After drying at 37°C for 48 h samples were weighted again and the solubility percentage was calculated as: $\%S = \frac{Wi - Wd}{Wi} \times 100$ where Wd is the dried sample weights after dissolution test. Each test consisted of four replicate measurements.

6.2.3 Cell culture

Human neuroblastoma SH-SY5Y cells (Cell Factor-IST ,Genova) were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. Experiments were performed in presence of Retinoic Acid (RA) in the culture medium after 24 h from seeded to differentiate SH-SY5Y cells and to promote extended neuritis. The cells were fed every 3 days and subcultured once they reached 80–90% confluence.

The morphology of the cells cultured on the different substrates was assessed by means of Scanning Electron Microscopy (SEM) and Laser Confocal Scanning Microscopy (LCSM).

Liver-specific cellular functions were investigated in terms of glucose consumption and lattate synthesis.

6.2.4 Cell fixation for SEM

Cells cultured on membranes, collagen and PSCD were prepared on day 7 and 16 for SEM analysis by fixation in 3% glutaraldehyde and 1% formaldehyde in PBS, followed by post-fixation in 1% osmium tetroxide and progressive ethanol dehydration.

6.2.5 Cell staining for LCSM

The morphological behaviour of cells was investigated with Laser Confocal Scanning Microscopy (LCSM, Fluoview FV300, Olympus, Milan, Italy) after the immunostaining of neuronal cytoskeleton and axon markers, βIII-tubulin and 43 KDa growth-associated protein (GAP-43), respectively . Six samples for each substrate were analyzed. In particular, neuronal cells were rinsed with PBS, fixed for 15 min with paraformaldehyde (4%), permeabilized for 10 min with 0.25% Triton X-100 and subsequently blocked for 30 min with 1% BSA at room temperature. To visualize βIII-tubulin a rabbit polyclonal anti-βIII-tubulin (1:100; Sigma, Milan, Italy) and a goat anti rabbit IgG FITC-conjugated (1:100; Invitrogen) were used. To visualize GAP-43, a monoclonal mouse anti-GAP-43 (1:100; Sigma, Milan, Italy) and a goat anti-mouse IgG TRITC-conjugated (1:100; Invitrogen) were used. Primary antibodies were incubated overnight at 4°C, secondary antibodies for 60 min at room temperature. Nucleic acids were counterstained with DAPI (200 ng/mL; Sigma, Milan Italy). Finally samples were rinsed, mounted and observed with a LCSM.

6.2.6 Metabolic assay

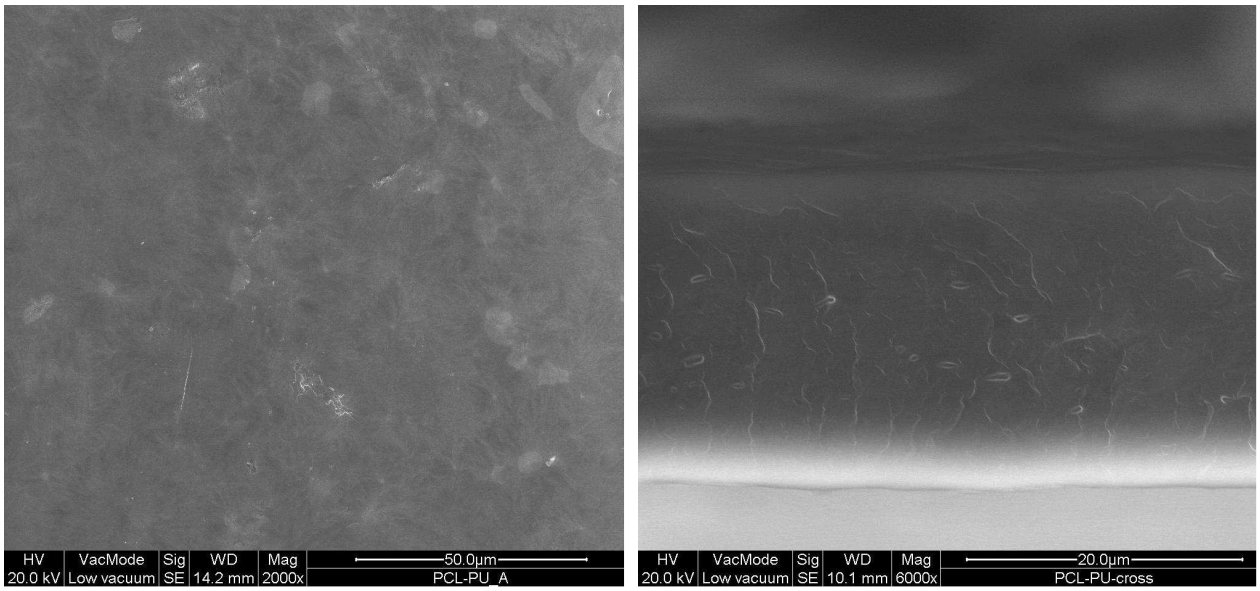
The metabolic activity of cells was evaluated by assessing glucose, lactate levels in the culture medium previously collected and stored in tubes at -20°C until assays. The glucose concentration in the medium was detected by using Accu-Chek Active (Roche Diagnostics, Monza Italy). Lactate

content was determined using lactate oxidase enzymatic assay Lactate Dry-Fast (Sentinel, Milan, Italy) via spectrophotometer analysis.

The statistical significance of all experimental results was established by using ANOVA test followed by Bonferroni t-test ($p < 0.05$).

6.3 Results

The PCL-PU membrane produced by inversion phase method has a complex structure as seen in the SEM picture (fig.2, 3)



Figures 2,3 : SEM picture of PCL/PU membrane surface and membrane cross section.

The surface shows a microstructure due the presence of PCL added during fabrication and spontaneously rise and dominate the surface. The PCL, used at low molecular weight, concentrated at the surface after blending homogeneously with a polyurethanes. The PCL component on the surface may confer improved neuronal compatibility to the polyurethane. This is showed in the contact angle measurements (fig. 4).

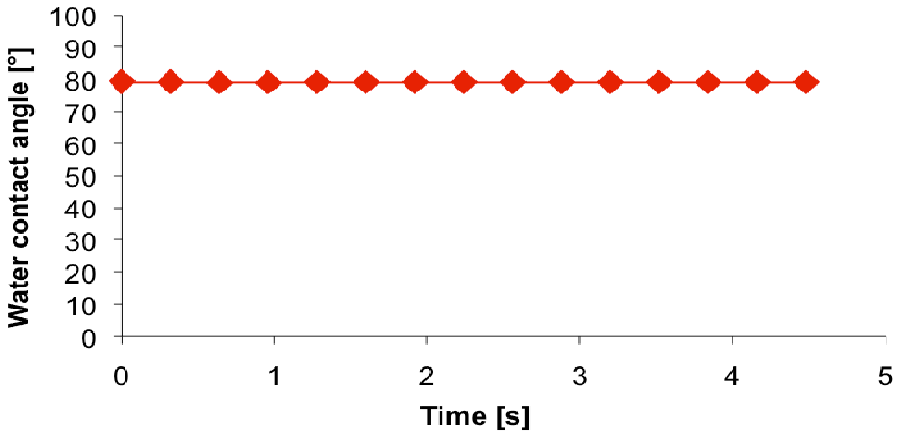


Figure 4: Water Contact Angle measurement in the time of PCL/PU membrane.

The addition of PCL to the PU solution given to the membrane the contact angle value 78,4° ± 1,5 like the contact angle value of polylysine, natural substrate of neuronal tissue.

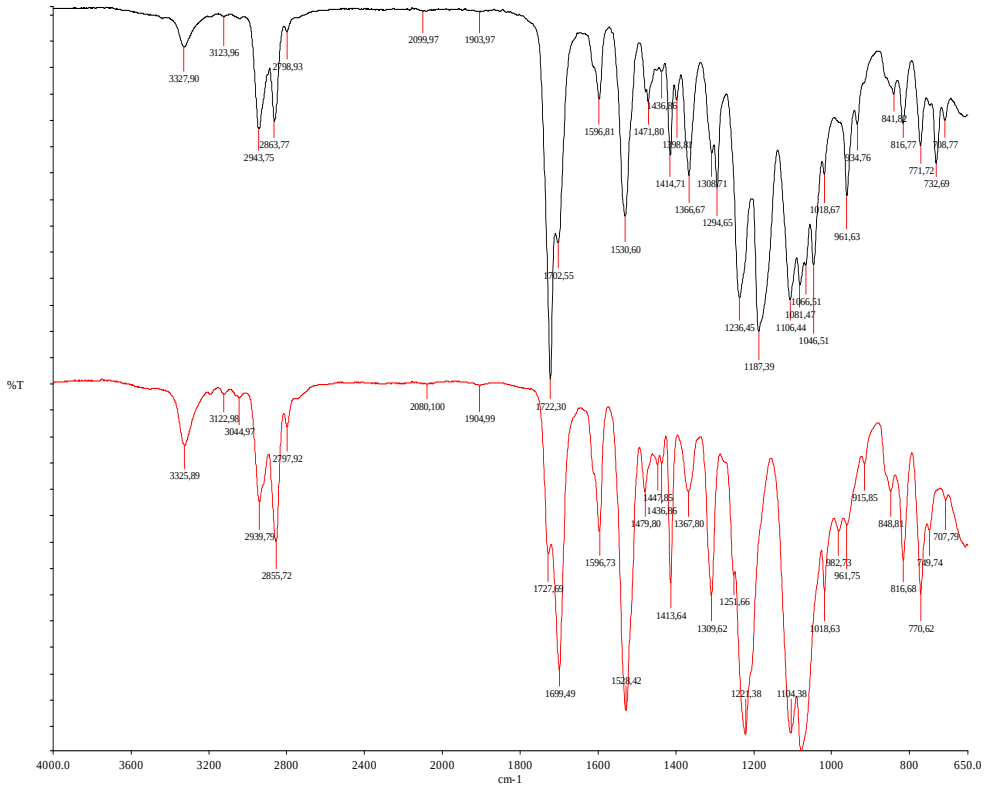


Fig. 5 : ATR spectra of PCL/PU membrane and PU membrane.

Fig.5 presents IR spectra of PCL/PU and PU membranes, respectively. Several characteristic bands located at 2943, 1722,1236 and 1187cm $^{-1}$are belonged to ester groups of PCL. Two typical bands at 1699 and 1100 cm $^{-1}$ for ether groups of polyurethane are recorded, respectively. It can be observed in Fig. 4 that several clear changes occur in the spectra of two membranes. Hydrogen bonding exists in polyurethanes, and involves the amide group as the donor, and the urethane carbonyl, the ether oxygen, or the carbonyl (in PCL) as the acceptor. All these registered events indicate that there are possible some interactions among the amin, carboxyl, and hydroxyl groups of two components inside the blend membrane. These interactions should be attributed to the hydrogen bonds possibly formed between amino and carboxyl groups, because there are no formation of new covalent bonds observed between PCL and PU chains based on these IR spectra. This result suggests that the degree of microphase separation in the PCL/PU membrane is the highest in the samples prepared. ATR result is in good agreement with DSC data (Fig.6).

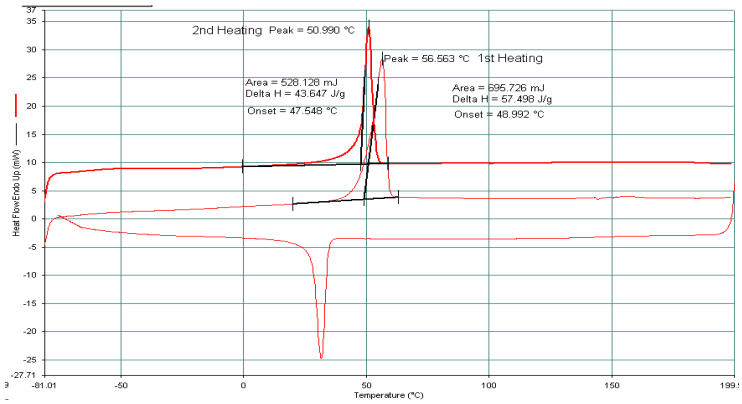
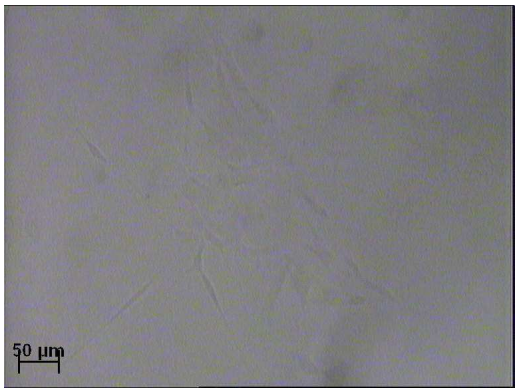


Fig. 6 DSC traces of PCL/PU membrane**.**

In principle, if two components are well blended together and completely miscible each other, only one new T_g would be observed between the original T_gs of components in the DSC thermogram of the blend; if they are partially miscible, the resulting blends would have two T_gs related to each component, but these measured T_g values corresponding to each component could be affected each other, depending on the composition ratios [13]. Fig 6 presents DSC thermogram for PCL/PU membrane. The melting point (T_m) of PCL can be easily around 60°C. A peak near 150° (almost

invisible) can be attributed to the PU. PCL and PU membrane did not show any features in their DSC curve with which their T_g s can be associated. PU is a semi-crystalline polymer due to its strong inter-and intra- molecular hydrogen bonds, and meanwhile, it also has a rigid amorphous phase because of its cyclic units. As a results, when PU is heated within a certain temperature range below its decomposition temperature, the variations in heat capacity related to the change in specific volume near T_g are probably too small to be detected by the DSC technique. It is known that the T_g of PCL is around -60°C. There is no any thermal event registered for the T_g of PCL in fig.6, although the T_m of PCL is moved to a lower value with a difference more than 3-4 °C. The T_m of PCL component, probably decreases as the weight ratio of PU increases. These trends are basically in agreement with reported results.



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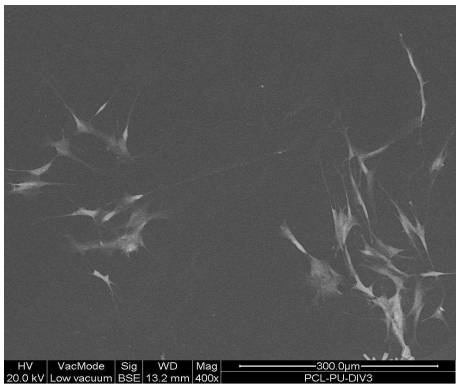
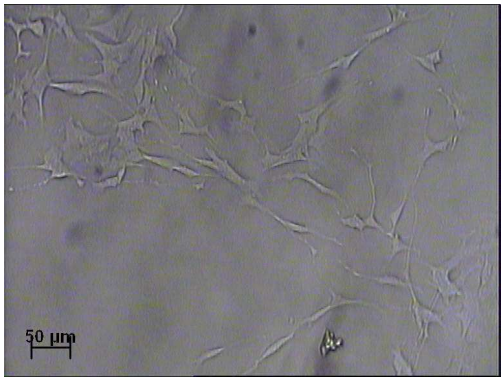


b)



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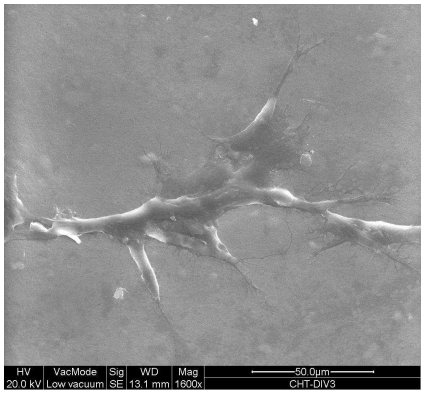

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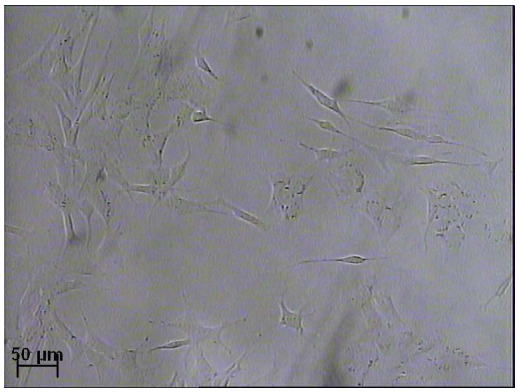

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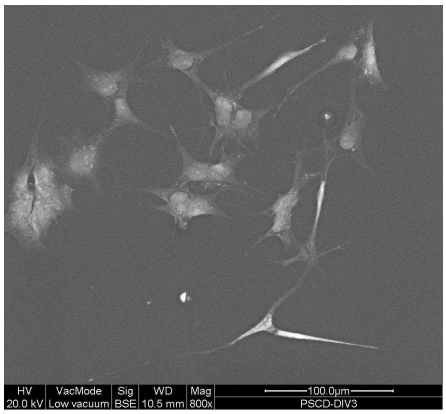
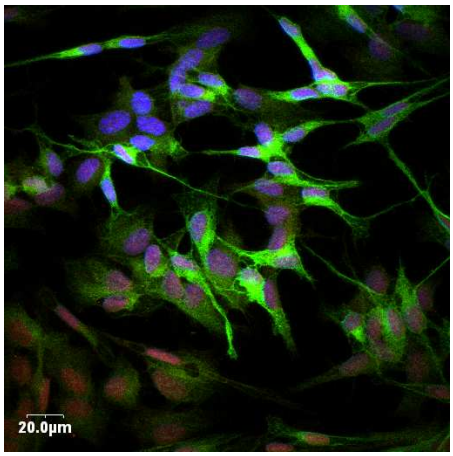


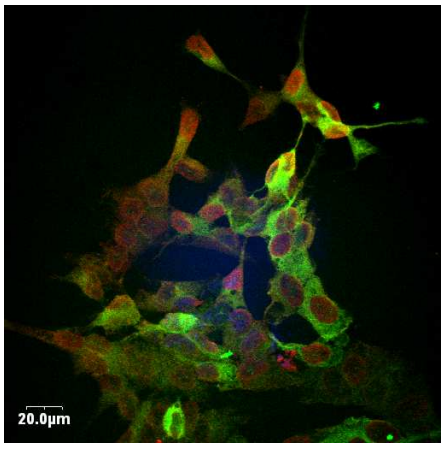
f)

Figure 7: Pictures of SHSY5Y cells on PCL/PU membranes (a, b); on CS membranes(c,d); on PSCD (e,f).

As shown by pictures, cells readily adhered to the PCL/PU membrane, polarity first, becomes evident, cells appear polarized, and cell's processes will become the axon and dendrites (Fig.7). Axons and dendrites continue to grow at rapid rate, but the remaining minor processes undergo little net elongation. The neuronal maturation is highly dependent on cell interactions with the substrate; pictures demonstrate PCL/PU membrane premise the neuronal development.



a)



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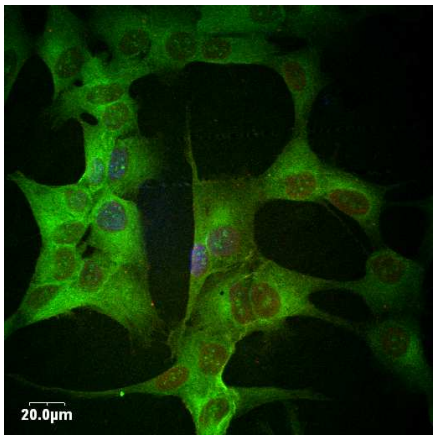

c)

Figure 8: Laser Confocal Scanning Microscope pictures of SHSY5Y on PSCD(a),CS(b) and PCL/PU(c) membranes.

The confocal images of the neurons, showed the localization of βIII-tubulin (green) in the neuronal network (Fig.8). This cytoskeletal protein was present in the soma and in all neuronal processes, while the axonal growth cones were visualized through the localization of GAP-43 (red), a specific protein involved in the regulation of axonal outgrowth.To emphasize this important result, the morphology of cells was analyzed, by measure of size of cellular body and neurites outgrowth (Fig.9).

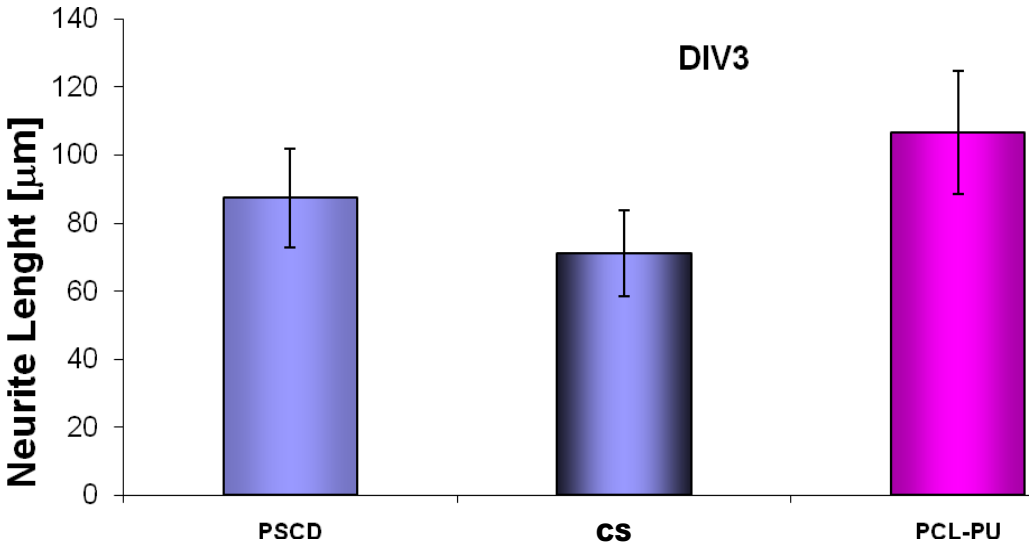

Figure 9: Average neurite lenght of SH-SY5Y after 3 day of culture (DIV3) on membrane CS, PCL-PU membranes and PSCD. The value are the mean of 5 experiments.

There aren't statistically significant differences on the glucose consumption between PCL/PU, CS to respect PSCD. The glucose consumption remains constant throughout the culture period (Fig.10).

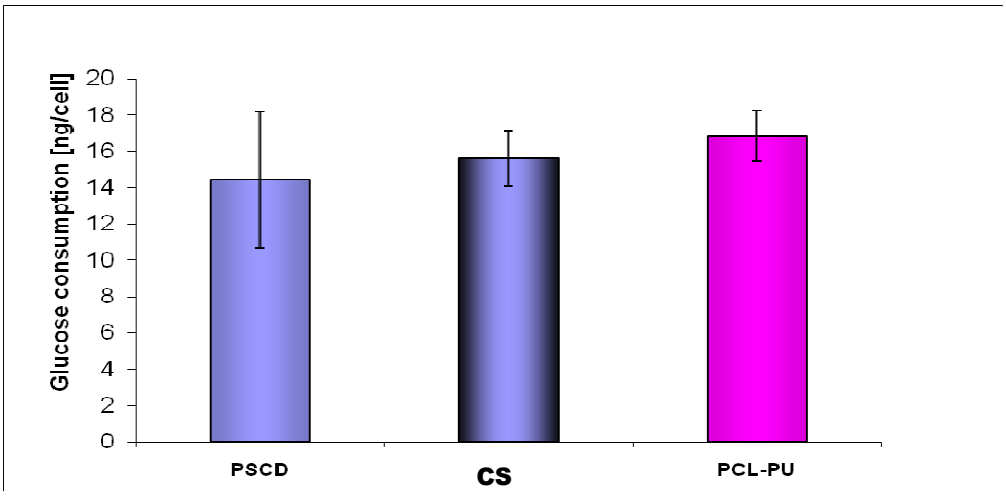

Figure 10: Glucose consumption of SH-SY5Y cells after 3 days of culture(DIV3) on CS, PCL-PU membranes and PSCD. The values are the mean of five experiments.

The lactate production rate was lower on PCL/PU and CS then PSCD throughout the culture period, suggesting a much better oxygenation of the cells on both membranes (Fig.11).

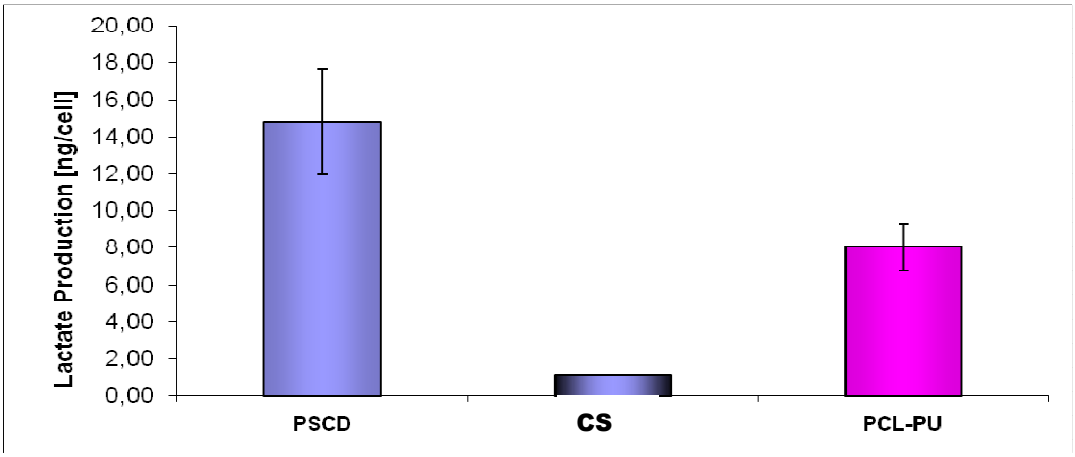

Figure 11: Lactate production of SHSY5Y cells after 3 day of culture on CS, PCL/PU membranea and PSCD. The values are the mean of five experiments.

The evaluation of the metabolic activity showed that cells maintained their functions at high levels throughout the entire culture period. This demonstrates PCL/PU support cell viability and functions.

6.4 Discussion

In the last few decades a variety of potential natural and synthetic biomaterials with various molecular designs creating a favourable microenvironment for nervous regeneration have been proposed to bridge the gap between severed peripheral nerve stumps. An appropriate nerve conduit must be biodegradable and must be exhibit good biocompatibility with extremely low inflammatory, immunogenic, and cytotoxic responses. In addition, the mechanical properties of the nerve conduit must guarantee that it does not collapse during the patient's movements but at the same time is sufficiently elastic to avoid tensions in the lesion site.in addition, it is desiderable that the conduit present a membrane thet mimics the size scales of fiber composing the extracellular matrix (ECM) of native tissue and organs, being permeable to the entry of nutrients into the conduit

lumen but presenting the necessary barrier to prevent the infiltration of unwanted tissues into the conduit from outside. These and additional characteristics have been recently introduced by innovative polymeric membranes approaches, which allow the design and modification of suitable biomaterials for the scope of nerve cell migration, proliferation, and differentiation on the injured site and subsequent nerve regeneration.

Microscale characteristics should be considere when designeg scaffolds for tissue engineering. These microstructural characteristics can selectively activate genes and mosulate cellular behaviour with regards to proliferation and differentiation[14,15]. However, the influence of the microstructure on SHSY5Y differentiation remains poorly understood.instead, the development of biomimetic scaffolds to enhance neuronal differentiation has largely focused on altering the chemical composition rather than the structural characteristics of scaffold. For example, the incorporation of collagen I to polycaprolactone was found to enhance differentiation of mesenchymal stem cells [16]. This evidence provided the rationale behind our efforts to produce a scaffold that would provide both chemical and biophysical lieage-specific cues similar to those of native extracellular matrix. The biophysical cues provided by the local microstructural environment rely primarily on interactions between the matrix and cell surface receptors, initiating intracellular signals mediated via cytoskeletal networks [17].

Our results demonstrate the PCL/PU membrane are able to promotes survival and neurite outgrowth of human neuroblastoma cells.

The cell biocompatibility of PCL/PU was tested by neurite extension of neurons in culture. SHSY5Y cells establish axons and dendrites during the time of culture both on PCL/PU and CS membranes. The density of axonal network increases with the time, and neurites become more elaborate and highly branched on the both substrates.

Neuronal cells on both membrane, displayed the typical morphology of hippocampal neurons that included a primary apical axon and several dendrites with multiple ramifications, developed from cell body (soma). There were no statistically significant differences between PCL/PU, CS membranes and PSCD in the neurites length (Fig.9).This results demonstrate that PCL/PU and CS membranes seem to be more supportive of neurite outgrowth modulating the development process of the neurons. Neurons have been shown to adhere, and growth on polymer by surface modification, with proteins such as fibronectin a vitronectin. This study shows that SHSY5Y cells can growth and differentiate, extending neurites, on biocompatible surfaces without protein coating. The correct and stable neuronal formation is supported by the evaluation of some major cytoskeletal

markers such as βIII-tubulin and GAP-43. Indeed, the constantly intense distribution of βIII-tubulin, specific for neuronal processes and soma structured neuronal networks. The metabolic functions of neurons on membranes were expressed at high levels demonstrated that, cells adhered on the membranes are functionally active for entire time of culture. The glucose uptake is similar on PCL/PU and CS to the PSCD culture, while lactate production on PCL/PU membranes is less to PSCD, demonstrated that the cells adhering on Petri dishes consume nutrients in a less grade of oxygenated medium to the PCL/PU membranes. The results demonstrated that the PCL/PU membrane improves the oxygen uptake better than PSCD culture.

6.5 Conclusion

Human SHSY5Y cell attachment and proliferation are comparable between PCL/PU and CS membranes. However, glucose comsuption and neurite growth are improved on PCL/PU when compared to CS membranes. These results support the concept of biomimetic scaffold as a novel strategy to promote proliferation of neuronal cells. These findings are promising and encourage a mechanistic investigation of the microstructural characteristics of PCL/PU membranes and their effects on peripheral neuron cells, possibly through cytoskeletal organization.

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