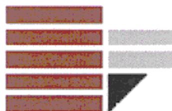

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Engineering membrane biohybrid system for hippocampal neuronal cells culture

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Publications

This thesis has resulted in the following book chapter, papers and conference proceedings:

Book Chapter

1. L. De Bartolo, **M. Rende**, G. Giusi, S. Morelli, A. Piscioneri, M. Canonaco, E. Drioli. "*Membrane bio-hybrid systems: a valuable tool for the study of neuronal activities*". In: M. Canonaco and R.M. Facciolo (Eds.) Evolutionary Molecular Strategies and Plasticity 2007; Research Signpost, pp.379-396.

Journal papers

1. L. De Bartolo, **M. Rende** S. Morelli, G. Giusi, S. Salerno, A. Piscioneri, A. Gordano, A. Di Vito, M. Canonaco, E. Drioli. "*Influence of membrane surface properties on the growht of neuronal cells isolated from hippocampus*". Journal of Membrane Science. 2008; 325: 139-149.
2. S. Morelli, S. Salerno, A. Piscioneri, **M. Rende**, C. Campana, E. Drioli, L. De Bartolo. *Membranes in regenerative medicine and tissue engineering*. In: L. Giorno and E. Drioli (Eds.) Membrane Operations; Wiley VCH, accepted
3. **M. Rende**, S. Morelli, G. Giusi, S. Salerno, A. Piscioneri, A. Gordano, M.Canonaco, E. Drioli. *Effect of Membrane Surfaces on Hippocampal Neuronal Cell Differentiation*. Tissue Engineering 2008; 725-726.
4. G. Giusi, R. M. Facciolo, **M. Rende**, R. Alò, A. Di Vito, S. Salerno, S. Morelli, L. De Bartolo, E. Drioli and M. Canonaco. *Distinct GABAA a*

receptor subunits exert early neurogenic activities on hippocampal cells. (Submitted).

Selected Refereed International Conference

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2. **M. Rende**, S. Morelli, G. Giusi, S. Salerno, A. Piscioneri, M. Canonaco, E. Drioli and L.De Bartolo. *Effect of membrane surface on hippocampal neuronal cell differentiation.Tissue Engineering and Regenerarive Medicine International Society,*TERMIS-EU, June 22-26, 2008, Porto, Portugal.

3. L. De Bartolo, **M. Rende**, G. Giusi, S. Morelli, A. Piscioneri, M. Canonaco and E. Drioli. The 6th International Membrane Science and Technology Conference, IMSTEC 07, 5-9 November 2007, Sydney (Australia).

4. L. De Bartolo, **M. Rende**, G. Giusi, S. Morelli, A. Piscioneri, M. Canonaco and E. Drioli Neuronal Membrane Bio-Hybrid System. 21th European Conference on Biomaterials, September 09-12th, 2007, Brighton, UK

Abstract

Abstract

Engineering membrane biohybrid system for hippocampal neuronal cells culture

L'obiettivo di questo lavoro di tesi è stato lo sviluppo di un sistema bioidrido a membrana, come modello di ricostruzione in vitro di tessuti neuronali per lo studio dei meccanismi di autogenerazione su differenti substrati.

Le membrane polimeriche semipermeabili, per le loro caratteristiche di separazione, immunoprotezione e di matrice artificiale possono essere adoperate per la ricostruzione dei tessuti e organi *in vitro*. La disponibilità di membrane in diverse configurazioni e in diverso materiale polimerico ha reso sempre più attraente e interessante il loro impiego nello sviluppo di nuovi tessuti nel settore biomedicale e diagnostico. Oggigiorno, le membrane possono essere usate per lo sviluppo di organi bioartificiali e per la ricostruzione di nuovi tessuti. Nonostante il crescente miglioramento ed i continui progressi delle tecnologie biomediche, la sostituzione di organi danneggiati da traumi e/o malattie rappresenta un problema cruciale per la moderna medicina. Le terapie attualmente in uso non solo sono estremamente costose, ma spesso non sono in grado di soddisfare pienamente gli scopi per i quali vengono applicate; il trapianto d'organi è severamente limitato dall'insufficienza dei donatori e dai problemi di compatibilità. Il superamento di queste difficoltà sembra sia possibile grazie ai continui sviluppi ottenuti in un settore di ricerca, emerso di recente nel campo delle scienze dei biomateriali, l'ingegneria tissutale o *tissue engineering*. In tale ambito, sono state messe a punto tecniche che permettono di coltivare in laboratorio linee cellulari e tessuti con le caratteristiche del ricevente [De Bartolo et al., 2007]. *In vivo,* le cellule sono supportate da una matrice extracellulare che influenza la loro morfologia, proliferazione e differenziazione nonché la loro funzione metabolica. Quando le cellule sono coltivate *in vitro,* un simile supporto meccanico e chimico deve essere fornito dall'ambiente di coltura, per questo motivo è molto importante conoscere le proprietà di membrana (chimico-fisiche, strutturali e di trasporto), che possono influenzare le funzioni che la membrana svolge e soprattutto la compatibilità del materiale polimerico a contatto con le cellule e i fluidi del corpo. Le cellule ed i tessuti

ottenuti *in vitro* sono poi innestati nel paziente, ripristinando le funzionalità compromesse, senza dover ricorrere al trapianto di elementi biologici prelevati da donatori estranei. I tessuti ingegnerizzati, in caso di successo, si integrano con quelli del paziente, apportando in tal modo un contributo specifico e duraturo alla cura dello stato patologico, senza richiedere debilitanti e costosi trattamenti farmacologici. In questa logica, la tecnologia è interessata allo sviluppo e alla produzione di nuovi sistemi per la coltura *in vitro* di cellule su larga scala, con particolare attenzione al controllo dei fenomeni e delle condizioni operative che regolano il trasporto dei nutrienti e dei prodotti di scarto del metabolismo cellulare. A tale proposito, numerosi studi sono stati condotti negli ultimi anni riguardo la possibilità di sviluppare sistemi artificiali basati sull'utilizzo di biomateriali, scaffolds e cellule, che consentano la sostituzione di tessuto nervoso danneggiato o permettano di ripristinarne l'organizzazione strutturale e anatomica e, conseguentemente, le capacità funzionali [Lee et al., 2003; Schmalenberg et al., 2005]. Tra i biomateriali impiegati nel campo della *tissue engineering*, le membrane polimeriche semipermeabili sembrano fornire il supporto meccanico e chimico necessario a garantire la regolazione del processo di crescita e di differenziamento delle cellule neuronali in sistemi bioibridi: le interazioni cellula-substrato inducono risposte cellulari specifiche, consentendo ai neuroni di assumere un definito orientamento nello spazio e la formazione *in vitro* di un ricco network di connessioni sinaptiche. Un sistema bioibrido a membrana adoperante cellule neuronali potrebbe, dunque, costituire un valido strumento nel campo dell'ingegneria tissutale, per consentire lo studio dei meccanismi molecolari alla base di malattie neurodegenerative e nello sviluppo di bio-molecole da impiegare nella terapia farmacologia, per consentire di ripristinare le funzionalità danneggiate [Simonin et al., 2006].
Lo stato dell'arte riguardante la coltura di cellule neuronali su matrici sintetiche, racchiude per la maggior parte lavori che riguardano la coltura di cellule tumorali, come linee immortalizzate. Tuttavia queste cellule hanno un metabolismo che risulta essere diverso dalle cellule primarie; di conseguenza le informazioni che si ottengono non possono essere trasportate completamente alla situazione in vivo.

Sono molti i casi in cui, per l'espansione *in vitro* di cellule neuronali, vengono utilizzate altre cellule di sostegno (glia, astrociti), allo scopo di supportare la crescita delle cellule di interesse (cocoltura). La cocoltura risultante può avere molti vantaggi rispetto alla coltura pura, in quando le cellule neuronali, ricevono naturalmente le sostanze necessarie per la crescita dalle cellule che in condizioni naturali fungono da sostegno.

Dallo studio di lavori su neuroni ippocampali, è possibile evidenziare che nella maggiorparte dei casi, sono stati valutati gli effetti del substrato sulle cellule, per pochi giorni di coltura (4-5 giorni); al contrario, è proprio dal 4° al 8° giorno di coltura che le cellule neuronali ippocampali, dopo la prima fase di adesione, iniziano a crescere aumentando i prolungamenti assonici e dendritici ed esplorando così l'ambiente circostante. L'adesione è importante nelle prime ore di coltura, perché indica la presenza di una buona superficie di attacco, cioè di una buona compatibilità del supporto, ma la differenziazione e vitalità a lungo termine può essere valutata solo allungando il periodo di osservazione della coltura oltre il 4° giorno. Durante la prima fase di crescita la cellula neuronale, essendo totipotente, riesce ad adattarsi all'ambiente circostante differenziandosi, grazie anche alla presenza di molecole importanti per la crescita nel mezzo di coltura. Solo successivamente alla formazione del network, si vengono a formare degli scambi molecolari con l'ambiente circostante, importanti per il mantenimento della vitalità e funzionalità cellulare a lungo termine.

L'ippocampo è un particolare zona del cervello che ha funzione di apprendimento e di memoria, risulta essere associata alla motivazione, al controllo delle emozioni, alla memoria e, oltretutto gioca un importante ruolo nel controllo delle risposte dell'organismo allo stress. Questa caratteristica plasticità è molto interessante soprattutto in caso di patologie come l'Alzahimer o in altre malattie degenerative, oppure nel coma.

Lesioni provocate all'ippocampo danno luogo a disturbi della memoria e dell'apprendimento piuttosto intensi. Sono stati testati su cavie, diversi farmaci che migliorano le funzioni cognitive in caso di danni cerebrali, ma i risultati sono modesti e a volte i farmaci risultano nocivi. Attualmente esistono evidenze sperimentali che i fattori ambientali giocano un ruolo fondamentale nel recupero delle capacità cognitive.

Essendo il numero di cellule neuronali stabilito alla nascita, e non variabile in quando i neuroni non hanno capacità di dividersi e proliferare, nasce l'esigenza di studiare i meccanismi di autogenerazione dei tessuti allo scopo di superare queste patologie e disturbi post-traumatici molto diffusi nella popolazione.

L'obiettivo di questo lavoro di tesi sperimentale è stato rivolto alla possibilità di realizzare sistemi bioibridi a membrana per la coltura di neuroni, isolati da una regione cerebrale coinvolta in importanti funzioni neurofisiologiche, quali l'apprendimento e la memoria: l'ippocampo. Numerosi lavori riportano l'utilizzo di tale regione cerebrale per gli studi neurobiologici e funzionali, valutando non solo stadi di sviluppo critici *in vivo* ed *in vitro* [Fukata et al., 2002], ma anche i meccanismi cellulari che sono alla base di svariate patologie neurodegenerative quali l'epilessia [Ullal et al., 2005]. Inoltre i neuroni ippocampali hanno una forma ben definita, facilmente monitorabili *in vitro* anche per lunghi periodi di coltura [Bunker and Goslin, 1998]. Il modello animale prescelto è stato l'ibernante facoltativo *Mesocricetus auratus*, ampiamente utilizzato negli studi neurodegenerativi, in quanto alcuni stadi di ibernazione ed in particolare il risveglio, rappresenta condizioni simil-ischemiche cui l'animale risponde con strategie di adattamento e plasticità sinaptica [Canonaco et al., 2005, 2008]. In tali sistemi la capacità delle membrane di fornire un adeguato microambiente alle cellule in coltura, dipende dalle proprietà morfologiche e chimico-fisiche della superficie di membrana e dalle proprietà di trasporto.

In una prima fase è stata sviluppata una nuova membrana piana, preparata mediante inversione di fase, a partire da un blend polimerico di polyetheretherketone (PEEK-WC) modificato e poliuretano (PU). Questa membrana offre il vantaggio di combinare le proprietà di entrambi i polimeri (biocompatibilità, resistenza termica e meccanica ed elasticità) con l'elevata proprietà di permeabilità, selettività ed una ben definita geometria dei pori della membrana. La membrana di PEEK-WC-PU è stata usata per la coltura di neuroni ippocampali in un sistema bioibrido allo scopo di rigenerare il tessuto in vitro. Per dimostrare la validità del sistema sperimentale, sono state determinate il grado di interazione e differenziazione cellulare e l'attività metabolica (cosumo di glucosio

produzione di lattato). I risultati preliminari mostrano la capacità di adattamento delle cellule neuronali ippocampali in coltura nel sistema bioibrido a membrana. Simile al comportamento delle cellule neuronali sulla polilisina, su questo tipo di membrana le cellule aderiscono e si differenziamento dando origine ad un complesso neuronal network. Come ulteriore conferma dell'avvenuto differziamento cellulare è stato utilizzato un marcatore cellulare specifico (MAP2), allo scopo di dimostrare l'inaterata struttura del citoscheletro delle cellule neuronali. I risultai di questo studio incoraggiano lo sviluppo di sistemi bioibridi a membrana per la coltura di neuroni ippocampali allo scopo di rimodellare e rigenerare *in vitro* tessuti in un microambiente controllato.

Le proprietà morfologiche del substrato possono svolgere un ruolo importante nell'interazione cellulare e soprattutto nel caso di cellule neuronali possono guidare topograficamente lo sviluppo di processi assonici e dendritici. A tale scopo in una seconda fase, è stato studiato l'effetto delle proprietà morfologiche della superficie di membrana, sul differenziamento delle cellule neuronali.

Membrane commerciali sia microporose di polietersulfone (PES) e poliestere (PE) e sia dense di fluorocarbone (FC) sono state caratterizzate allo scopo di definire le loro proprietà morfologiche (e.g., dimensione media dei pori, porosità, rugosità, distribuzione della dimensione dei pori) e le loro proprietà chimico-fisiche (e.g. bagnabilità, adsorbimento di acqua).

Le membrane sono state modificate con coating di polilisina allo scopo di favorire l'adesione cellulare e di creare superfici con gli stessi gruppi funzionali interagenti con le cellule ed investigare solo l'affetto della morfologia di superficie sul differenziamento cellulare. Le membrane modificate sono state caratterizzate per valutare l'uniformità del coating, la rugosità della superficie e le proprietà di trasporto. Il coating ha modificato le proprietà chimico-fisiche in termini di bagnabilità, uniformando le differenze superficiali delle membrane native. Le membrane così modificate presentavano una rugosità si superficie compresa tra 6-200 nm. La modifica di superficie ha ridotto la permeanza idraulica delle membrane di poliestere e di polietereterchetone rispettivamente del 60% e del 25%, mentre per le membrane di polisulfone è rimasta invariata.

Lo sviluppo ed il differenziamento delle cellule in coltura sono stati valutati in termini qualitativi, attraverso l'osservazione dei cambiamenti morfologici dei neuroni isolati e della formazione di un caratteristico network di prolungamenti assonici e dendritici sia sulle membrane di PEEK-WC che su membrane commerciali, quali PE, PES, FC. E' stata valutata quindi la localizzazione e la distribuzione di marcatori strutturali come la β-tubulina, una proteina associata al citoscheletro, presente nel soma e in tutti i prolungamenti neuronali. Per favorire la visualizzazione dei prolungamenti assoni è stata scelta una proteina specifica di tale prolungamento e precisamente la *Growth-Associated Protein-43* (GAP43).

Il metabolismo delle cellule è stato valutato in termini di consumo di glucosio e produzione di lattato, presenti nel mezzo di coltura. Inoltre è stata valutata la secrezione di fattori neurotrofici come il *Brain Derived Neurotrophic Factor* (BDNF), al fine di analizzare l'attività neuronale di sintesi di specifici fattori differenziativi durante lo sviluppo *in vitro*. E' stato in tal modo investigato il mantenimento a lungo termine della vitalità e della funzionalità dei neuroni per un periodo di tempo di 12 giorni di coltura.

La rugosità della superficie di membrana ha influenzato notevolmente l'adesione cellulare e la formazione del network meuronale sulle membrane di FC (Ra= 6nm) e di PES (Ra = 50 nm). Le cellule formano prolungamenti assonici e dendritici che si ramificano e si connettono con i prolungamenti in un network molto complesso. Al contrario in membrane più rugose come quelle di PEEK-WC (Ra= 199,2) le cellule tendono ad aggregarsi ed a formare prolungamenti che si sviluppano nei pori della membrana. Le osservazioni microscopiche sono state confermate da analisi quantitative della lunghezza assonica e del metabolismo cellulare. Sulle membrane con rugosità compresa tra 6 nm e 50 nm i prolungamenti assonici sono significativamente più lunghi rispetto alle membrane con una rugosità maggiore. Le membrane con una rugosità compresa tra 6-50 nm favoriscono la formazione di strutture neuronali polarizzate e la produzione di neurotrofine specifiche come il BDNF, che ha un ruolo nella sopravvivenza e maturazione di specifiche popolazioni neuronali nonché nella trasmissione sinaptica.

Il sistema bioibrido costituito da membrane i FC e neuroni ippocampali è stato adoperato per studiare l'azione modulatrice delle sub unità α dei recettori del acido γ-Aminobutyric type A (GABA), sull'organizzazione dei processi neuronali. A tale scopo sono stati adoperati molecole che svolgono un'azione altamente agonista sulle subunità α_2 e molecole che svolgono un'azione antagonista sulle subunità α_5. Queste subunità grazie alla loro localizzazione sinaptica ed extrasinaptica, sono critiche per le funzioni immunogeniche associative, da cui si evince che, l'interazione tra il sistema GABAergico e il sistema Gluergico può costituire un elemento potenziale che è cruciale per regolare lo sviluppo assonale e dendritico.

Successivamente sono state sviluppate membrane a fibre cave (HFMs) di PEEK-WC e di Polyacrilonitrile (PAN) allo scopo di sviluppare un sistema tridimensionale altamente integrato. Le membrane in configurazione a fibra cava sono vantaggiose rispetto alle piane poiché offrono: i) Un'ampia superficie di adesione e di scambio di nutrienti ed un volume molto piccolo; ii) una compartimentalizzazione all'inerno e all'esterno della fibra e quindi separazione del compartimento cellulare da quello ex cellulare; iii) un'adeguata perfusione evitando fenomeni di shear stress.

Le HFMs sono state preparate mediante *dry-wet spinning* e successivamente caratterizzate allo scopo di conoscerne le proprietà chimico fisiche e di trasporto.

Le proprietà di permeabilità delle membrane sono particolarmente importanti per le fibre cave per lo scambio molecolare tra il compartimento cellulare e l'ambiente esterno. Quindi è stato determinato il cut-off delle fibre di PEEK-WC e di PAN che sono rispettivamente all'incirca di 78000 Da e 81000 Da. Le membrane modificate mediante coating con PLL sono state utilizzate nella coltura a lungo termine di neuroni ippocampali.

La capacità di adesione e accrescimento delle cellule è stata valutata per 12 giorni di coltura. Sulle fibre di PEEK-WC è stata osservata la formazione di un network neuronale piuttosto complesso ed omogeneamente distribuito in tutta la superficie esterna della fibra mentre sulle membrane di PAN le cellule hanno formato in diverse zone della superficie di membrana dei distretti cellulari dai quali si sviluppano i prolungamenti neuronali. Su entrambe le fibre, le cellule sono state funzionalmente attive per 12 giorni. Le fibre di PAN

hanno sviluppato un maggior consumo di glucosio e produzione di lattato rispetto alle fibre di PEEK-WC. Il costrutto tissutale sviluppato ha mantenuto la sua funzionalità per 12 giorni di coltura. Le membrane in configurazione a fibra cava hanno consentito lo sviluppo di un sistema tridimensionale che da un punto di vista microarchitettonico consente di dirigere e guidare la rigenerazione neuronale in un sistema modello *in vitro*.

Nell'ultima fase di questo lavoro è stato sviluppato un sistema bioibrido adoperando una membrana biodegradabile di chitosano.

I polimeri biodegradabili sono materiali che si decompongono ma i cui prodotti di degradazione persistono a lungo nell'organismo ospitante. Spesso questo termine include le sotto classi di materiali assorbibili, riassorbibili, bioassorbibili e biodegradabili.

Molti scenari di applicazione, compresa l'ingegneria tessutale, necessitano per l'utilizzo *in vivo* di matrici polimeriche biodegradabili o di strutture che siano minimamente soggette a interazioni con il tessuto in via di sviluppo. Lo sviluppo di queste diverse funzioni solitamente richiede una microstruttura di sostegno porosa, con caratteristiche di porosità proprie dell'applicazione specifica.

Il chitosano è un polisaccaride biosintetico che è ottenuto dalla deacilazione della chitina, che è un polisaccaride naturale prodotto da un'enorme numero di organismi viventi. Il chitosano è l'unico polimero pseudonaturale caricato positivamente e questo lo rende importante per molte applicazioni biomedicali.

L'adesione e la crescita delle cellule su questa membrana è stata valutata mediante osservazione della coltura nel tempo (16g) ed analisi dell'attività metabolica (cosumo di glucosio, produzione di lattato) e di sintesi (BDNF) dei neuroni ippocampali in coltura.

Le cellule neuronali ippocampali in coltura sulle membrane di chitosano aderiscono e si differenziano senza necessità di modifica della superficie di membrana attraverso l'utilizzo di biomolecole come la polilisina (PLL). La morfologia della cellula sulle membrane di chitosano del tutto simile alle cellule in coltura sul substrato di controllo (costituita da PSCD rivestito di PLL), non sono presenti differenze significative nella grandezza del soma e nella lunghezza dei neuriti. L'attività metabolica delle cellule, in termini di consumo di glucosio e produzione di lattato, viene mantenuta ad alti livelli per tutto il

periodo di coltura; esse inoltre esibiscono una maggiore capacità di sintesi della neurotrofina BDNF, conseguente ad un miglior grado di connessione sinaptica tra le cellule.
I risultati incoraggianti rappresentano un punto di partenza per lo sviluppo successivo di sistemi biobridi a membrane e biodegradabili in impianti tissutali e nella riparazione di tessuti. I risultati ottenuti rappresentano un primo approccio nella realizzazione di sistemi bioibridi che consentono una coltura di tipo tridimensionale e quindi paragonabile a quella *in vivo*. Tali sistemi, adoperanti neuroni ippocampali, possono essere utilizzati nello studio, *in vitro,* del comportamento morfologico e funzionale di popolazioni neuronali danneggiate in alcune delle più comuni malattie neurodegenerative, come il morbo di Alzheimer e l'epilessia [Ullal et al., 2005; Cotel et al., 2008].

Chapter 1

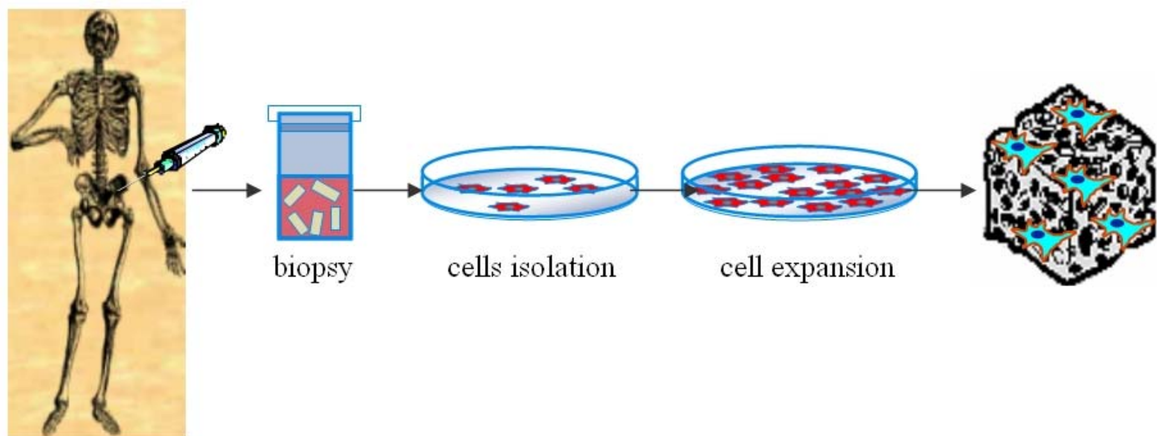
Chapter 1

Introduction

1.1 Research motivation: Tissue Engineering

In the last years the big progress of cellular's biology and biotechnology, has led to the development of news technologies for cells culture and in vitro regeneration of tissues and organs, a new field of the biomedical science knows as *"Tissue engineering"*. [1]

The goal of tissue engineering is the design and construction in the laboratory of living and functional components that can be used for the regeneration of malfunctioning tissues.



Although considered as a relatively new field, the first documented report of tissue engineering emerged in 1933, when tumor cells were entrapped in a polymer membrane and implanted into a pig. Tissue engineering is an interdisciplinary field that brings together the principles of life sciences and medicine with those of engineering and has three basic components: cells, supports and signals. Its development over the past decade has been the result of a variety of factors: increased knowledge and availability of cells, the advent of new biomaterials as potential templates for tissue growth, improvements in a bioreactor design and increased understanding of healing processes have all contributed. However, although tissue engineering research is evolving rapidly, there has been a hiatus in the commercial development and, hence, clinical application of engineered products.

However, progress continues and the number of people currently benefiting from tissues engineering is set to expand exponentially in the coming years [2].

1.1.2 The challenge

The challenge for tissue engineering is optimise the isolation, proliferation and differentiation of cells, and design of biohybrid systems that are able to support and coordinate the growth of three-dimensional tissues in the laboratory.

One idealistic strategy would be harvest stem cells from a patient, expand them in cell culture, and seed them in biohybrid systems. Stem cells can become many types of mature cells, via a process called differentiation, when given the specific biological stimuli. The biohybrid systems then act as template and stimulus for proliferation and differentiation of cells into the specific cells that will generate specific new tissue. The tissue can either be grown on a structure that will completely disappear (resorb) as the new tissue grows, so that only the new tissue will be implanted, or biocomposite of structure and new tissue can be implanted. The tissue engineered construct must be able to survive and restore normal function, e.g. biochemistry and both mechanical and structural integrity, and integrate with the surrounding tissues. Using cells from the same patient eliminates the problem of immunorejection that can occur with transplants from donors.

1.1.3 The cells sources

Probably the single most important element in the success of tissue engineering is the ability to generate appropriate numbers of cells (too many cells can be just as detrimental as too few) and the capacity for those cells differentiate from, and maintain, the correct phenotype and perform specific biological functions. For example, cells must produce an extracellular matrix in the correct organisation, secrete cytokines and other signalling molecules, and interact with neighbouring cells/tissues. Immediately, this raises a number of potential problems, not least of which is obtaining appropriate cell numbers to promote repair.

There are two main types of cell culture: one is primary culture and other continuous culture (cell lines). Primary cultures are obtained directly from animal or human tissues

and are cultured either as small pieces of tissues or single cells following isolation from the tissue by digestion enzyme of such as trypsin and collagenase. The main disadvantage of primary cultures is that they become senescents, lose their ability to multiply and may lose some phenotypic characteristics with time. The main advantage of primary cultures is that they retain many of their original characteristics in their limited life span. Continuous cells lines can be maintained in culture either for a limited number of cell divisions or indefinitely. Many of these cell lines are derived from cancerous tissues, while some of these cell lines are transformed into immortal cell using viral oncogenes. These continuous cell lines have the advantage of unlimited availability but have the disadvantage of preserving few of the original cellular characteristics. Cells derived from a source must attach to substrate in order to grow, while cells derived from blood grow in suspension. Cells in suspension have a round shape, and cells attached to substrate show different morphologies depending on their tissue of origin.

Once the cells attach to substrate, they start to divide and multiply to form a complete layer (commonly known as confluent layer) covering substrate.

1.1.4 Culture condition

Cells in culture require carbohydrates, salts, aminoacids, vitamins, fatty acids and proteins to survive in vitro. The basal medium contains the essential inorganic salts, aminoacids, vitamins, fatty acids and some proteins. It also contains phenol red and bicarbonate based buffering system and a combination of different antibiotics or anti fungal agents can also be used in cell culture to avoid contamination.

As indicated above, by manipulating the culture conditions, it is possible to control the available differentiation pathways and selectively generate cultures. Such manipulations include stimulation of cells with particular cytokines, growth factors, aminoacids, other protein, drug and co-colture with target cell/tissue type. Utilising these approaches is possible to know the different effects products [2].

1.1.5 Three-dimensional interactions

The great diversity in the structure and function of various tissues and organs, is a primary reason for the highly multisciplinary nature of tissue engineering. Tissues and organs consist of specialised living cells arranged within a complex structural and functional framework know as extracellular matrix (ECM). The normal function of most cells and tissues depends on spatial interaction with neighbouring cells and with a substratum or a matrix. Cell-cell and cell-ECM interactions are coordinated by members of several families of membrane spanning proteins, called adhesion molecules. These are fundamental to cell adhesion, helping to define 3-D cellular organization and also to participate directly in cell signalling, controlling cell recruitment, growth, differentiation, immune recognition and modulation of inflammation. Consequently, the function of the ECM and 3-D cell interactions is an important aspect of generating viable constructs for in vitro tissue regeneration. A number of natural and synthetic materials have been used to produce 3D structure to function as an artificial ECM. Materials should be non toxic, have good biocompatibility, be biodegradable and be capable of interacting specifically with the cell type of interest. Work with such materials has shown how materials can also be made to be bioactive through adsorption with biomolecules and that such modifications can enable specific recruitment and adhesion of specific cell types [2].

1.2 Objective and scope of this Thesis

Restitution of brain function following trauma or disease is a challenge for neurobiologist and neurobiotechnologist, because the central nervous system (CNS) cannot regenerate on its own. Attempts to replace lost or dysfunctional neurons by means of tissue transplantation or peripheral nerve grafting have been intensely investigated for over a century. These approaches have evolved as a powerful tool for restoring function in damaged or diseased regions of the CNS and also for addressing basic issues related to development function and plasticity in the CNS. As an alternative to conventional technologies, a new approach is being investigated which uses cell engineering derived

biomaterials to influence function and differentiation of cultured cells. Neuronal tissue engineering is an emerging field which derives from the combination of various disciplines that interact in order to study the development and regeneration of neuronal tissue. Neurodegenerative processes are becoming a major health concern especially since they are the cause of many neuronal disorders.

The goal of this work is to achieve a biohybrid system constituted by membranes and hippocampal neurons for in vitro regeneration of neuronal tissue.

The neuronal tissue engineering construct, is a novel approach to study at molecular level neurodegenerative disorders, pathogenic state as well as the development of new therapeutic molecules. Polymeric semi-permeable membranes furtherer characteristics of selectivity, biostability and biocompatibility appear to be promising biomaterials for *in vitro* cell growth. In fact membranes provide mechanical and chemical supports for the anchorage-dependent cells. They allow compartmentalization of cells providing a selective transport of molecules avoiding the passage of other species.

The use of polymeric semi-permeable membranes with different physico-chemical and transport properties is an appealing approach in the tissue and bio-artificial organ engineering field, since these bio-membranes share specific features such as the selective transport of molecules, resistance and protection and for the *in vitro* simulation of human brain functions substitute.

In a first phase, a novel flat membrane was prepared from a polymeric blend of modified polyetheretherketone (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 techniques. This membrane offers the advantage of combining the properties of both polymers (biocompatibility, thermal and mechanical resistance, elasticity) with those of membranes such as permeability, selectivity and well-defined geometry. This membrane is used for culture of hippocampal cells in a bio-hybrid system.

For typical neuronal tissue-engineered constructs, the properties of both cell (morphology, viability functions) and material (physico-chemical, morphological and transport properties) components are very important [3].

Subsequently the effect of morphological properties of membranes in terms of pore size, porosity and roughness on the adhesion and differentiation of hippocampal neurons was investigated. To this purpose, membranes with different surface properties were used. In particular commercial microporous membranes such as polyester (PE), polyethersulfone (PES), and dense membrane as fluorocarbon (FC) membranes. This lather membrane is interesting for its permeability properties to oxygen, carbon dioxide and aqueous vapour. Microporous membranes were also developed from polyetheretherketone (PEEK-WC).
These membranes used as substrates for cell adhesion are coated with poly-L-lysine (PLL) in order to have the same functional groups interacting with cells.
The behaviour of neurons isolated from the hippocampus on membranes with different surface properties was investigated in terms of cells growth parameters, and metabolic functions. Neuronal cells response to the different membrane surface was evaluated by analysing their morphology and neurite outgrowth as well as their specific metabolic functions as the neurotrophin secretion.
The developed bihoybrid system was used to evaluate the morphogenic role of γ-aminobutyric acid type A ($GABA_A$) neuroreceptors. Of the most common 19 $GABA_A$ receptor subunits, α subunit appears to be the main component largely responsible for inhibitory interneuronal functions; canonical elements that are mostly GABAergic in nature. The neurogenic role of $GABA_A$ α_2 and α_5 subunits on the receptors of the Gluergic neuronal system, which are evolved in developmental, synthetic plasticity and excitotoxicity events, was investigated by using highly specific α_2 agonist molecule, funitrazepan and α_5 selective inverse agonist ,RY-080.
Moreover, the optimization of transport, physico-chemical and structural properties of the membrane as well as fluid dynamics of cellular microenvironments could affect cell-membrane interactions and the functional maintenance of hippocampal cells.
Membranes in hollow fiber configuration are particularly useful to enhance the surface area for the adhesion of cells in a small volume. Hollow fiber membranes allow the compartmentalization of cells in the shell or in the lumen of the fiber that communicate through the pores present in the wall thickness with medium compartment.

In bio-hybrid systems using hollow fiber membranes made of modified Polyetheretherketone (PEEK-WC) and Polyacrylonitrile (PAN) were developed.
Since an efficient transport of metabolites and nutrients is required for *in vitro* maintenance of cell viability and functions, the hydraulic permeance and the mass transfer of metabolites through the membranes were evaluated.
The viability of hippocampal cells was tested by assessing the cell adhesion to the hollow fiber surface. The functional behavior was investigated in terms of glucose consumption, lactate production and synthesis of brain-derived neurotrophic factor (BDNF) [4]. The developed biohybrid system was used.
Finally, biologically derived a chitosan membrane, was proposed as a potential biomaterial for supporting both adhesion and differentiation of neurons *in vivo*. Chitosan is a biosynthetic polysaccharide that is the deacetylated derivative of chitin, a naturally occurring polysaccharide that can be extracted from crustacean exoskeletons or generated via fungal fermentation process. This polysaccharide was used for prepare a flat membrane with phase inverse technique. Chitosan membrane has been utilized for the long term culture of hippocampal cells.
The chitosan films showed a significant enhancement of neurite outgrowth, reflecting the dependence of neuronal cell affinity to the amine content in the polysaccharide, an important relation that is important for neuronal tissue engineering applications.
This biodegradable polymer should be applicable to those tissue engineering products for which tissue repair or remodelling is the goal.

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Chapter 2

Chapter 2

Background

2.1 Introduction

One approach to tissue engineering is to create an *in vitro* environment that embodies the biochemical and mechanical signals that regulate tissue development and maintenance in vivo. The in vitro tissue engineering system is composed of three major components:

(1) Metabolically active cells able to express their differentiated phenotype,

(2) Polymeric materials that provide a three-dimensional (3D) structure for cell attachment and tissue growth,

(3) Bioreactor culture vessels that provide an *in vitro* environment in which cell-polymer constructs can develop into functional tissues.

These constructs can potentially be used *in vitro*, for controlled studies of tissue growth and function, or *in vivo* for tissue repair.

In this chapter, want focus on these different contributes cells and polymeric membrane; both are based on engineered constructs.

2.2 Organ model used in this study

Cells types used for this study was obtained from hippocampus of 1-2 day-old neonatal *mesocricetus auratus* hamster (golden hamsters are hibernating rodents used as research animals); the *mesocricetus auratus* is an optional hibernator rodent, whose brain it is equipped of the ability to put into effect various neuroprotective mechanisms like the hypothermia, drastic reduction of the metabolism and increased defended anti-oxidant during the various stages of the process of hibernation [1]. This rodent can constitute therefore a useful model in the understanding of the molecular events to the basis of neurodegenerative pathologies, like the ischemia that, in hibernator, they can be manifested at the moment of the awakening [2] [3].

The Hippocampus is a part of the forebrain, located in the medial temporal lobe. It belongs to the limbic system and plays major roles in short term memory and spatial navigation.

Humans and other mammals have two hippocampi, one in each side of the brain. In rodents, where it has been studied most extensively, the hippocampus is shaped something like a banana [4].

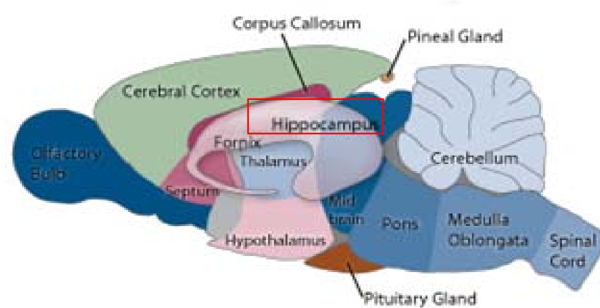


Fig.1 Bilateral section of mesocricetus auratus brain

In humans it has a curved and convoluted shape that reminded early anatomists of a seahorse. They are two parts: hippocampus major (or horn Ammon) and hippocampus minor, especially associated with memory.
The hippocampus has been the object of much experimental work, and theories about the nature of its function have multiplied. One possibility which has attracted attention from behaviourists is that it may be involved in memory. However, at present no clearly formulated results can be stated. This is consequence partly of the anatomical complexity of the region and partly of the semantic difficulties surrounding the words “memory” and “learning”.

2.2.1 Human hippocampus

The human hippocampus can be divided into three distinct fields, which we have labelled CA3, CA2, CA1 according to the nomenclature of Lorente de No’ (1934). Field CA3 borders the hilus of dentate gyrus, where it terminates in a complex fashion. At its border end, it borders field CA2. Cassell and Seress have estimated that there are about

$2.1*10^6$neurons in the CA3 region and about $0.22*10^6$ pyramidal cells in CA2. These regions of hippocampus contain pyramidal cells and a number of interneurons. Field CA1 is unquestionably the most complex subdivision of the human hippocampus. It appears to be populated by a far more heterogeneous group of neuronal elements than the other hippocampal fields. There are many connections between these fields that are important for the perfect features of this forebrain's part.

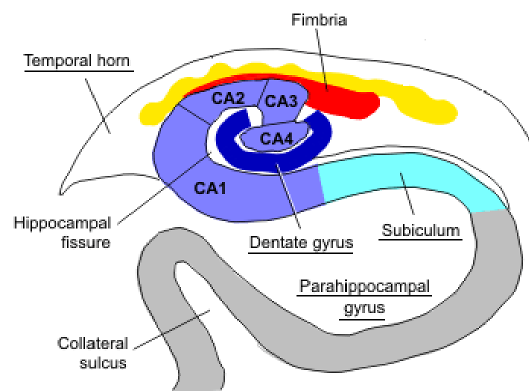


Fig.2 Cito-architecture of human hippocampus

2.2.2 Clinical disease

Various clinical conditions result in morphological alterations of the human hippocampal formation. While the causative factors are not yet know for most of these disease states, it is clear that each of the different hippocampal cytoarchitectonic fields is more or less vulnerable to damage. In ischemia and temporal lobe epilepsy, for example, field CA1 of the hippocampus suffers the greatest neuronal cell loss. In other neuropathological conditions while in the Alzheimer's disease, the hippocampus is one of the first regions of the brain to suffer damage; memory problems and disorientation appear among the first symptoms. Among the many conditions that produce pathological changes in the

hippocampal formation Alzheimer's disease is probably the most devastating. Myriad studies have come to clear conclusion that this disorder is distinctly different from an elaboration of normal aging. Alzheimer's disease is associated with four neuropathological correlates in the hippocampal formation: neuronal cell loss, neurofibrillary tangles, neuritic plaques, and granulovacuolar degeneration. The researches have conducted systematic analyses of cell loss in the hippocampal formation, and they estimate that there is decrease of some 56% in number of hippocampal pyramidal cells in the CA1 fields. As result of the cell loss and other pathological sequelae of Alzheimer's disease, the hippocampal formations becomes, in essence, functionally disconnected from its major afferent and efferent interactions. Given the important role the hippocampal formation is known to play in certain forms of memory, it is likely that a major portion of the problems the memory function observed Alzheimer's disease is attributable to damage of the hippocampal formation.

Damage to the hippocampus can also result from oxygen starvation (anoxia), encephalitis or medial temporal lobe epilepsy. Temporal lobe or complex partial epilepsy is another neurological disorder in which the hippocampal formation is severely affected. This most common form of epilepsy is generated from a dramatic loss of neurons in CA1 region during the hippocampal formation. In approximately two-thirds of cases of temporal lobe epilepsy, the hippocampal formation is only structure that shows pathological modifications, but in many cases cell loss in the CA3 portion. In some cases, also the organization of cell layer and metabolic alteration (hypoxia, anoxia, ecc.) influence the functions of neuronal cells, which are cause of this disease.

Multiple lines of research have recently converged on a critical role for the hippocampus in episodic memory, choice the ability to remember specific personal experiences (Tulving, 1983). Humans with selective hippocampal damage exhibit deficits in episodic memory, sometimes with relative sparing of the ability to acquire general factual knowledge, or semantic memory.

Patients with extensive hippocampal damage, like Ischemia, Schizophrenia, and other disorders, demonstrate anterograde memory impairment apparently resulting from the

hippocampal damage, may experience amnesia, that is, inability to form or retain new memories [5].

Know the tissues formation and the physiological and pharmacological developing of neuronal network, in vitro, can contribute to understand the causative factors of this pathological disease.

2.2.3 Cellular adhesion

A major goal of tissue engineering is to employ the principles of rational design to recreate appropriate signals to cells that promote biological processes leading to production of new tissues or repair of damaged ones. A key modulator of cell behaviour is the extracellular matrix (ECM) that provides individual cells with architectural cues of time and space, modulates bioavailability of soluble growth and differentiation factors, and organizes multicellular tissue development.

In vivo, cells are supported from one extracellular matrix that it influences the morphology, proliferation and metabolic functions. Moreover cells sense and response to a variety of signals that include those that is soluble such as growth factors, differentiation factors, cytokines, and ion gradients. In addition, cell behaviour and phenotype is governed by responses to other types of signals that include mechanical forces, electrical stimuli, and various physical cues. Immobilized protein matrices that generally are fixed in space also regulate cell function. The general term that has come to denote the complex mixture of proteins on outside of cells that governs their behaviour is the ECM. Evolution has provided cells with surface receptor to ECM components that enable them to recognize and decipher the signals that they encounter from the ECM and which influence cell growth, division and differentiation. [6]

For descriptive purposes, cell adhesion is classified into categories of cell-substratum and cell-cell attachment. Cell-cell interactions may occur between like cells (homotypic events) or between dissimilar cells (heterotypic events).

There exists in tissues an exquisite balance between the anabolic process of ECM production and the catabolic process of ECM turnover. Introduction of foreign materials

inevitably disrupts this natural homeostasis. A goal of tissue engineering is to successfully introduce the materials that will, through stimulation of anabolic processes, lead to ECM production and acceptance of engineered material. Achievement of this goal requires a through understanding of the structural relationships among molecules in the ECM, their molecular interactions directing cell adhesion events, their biosynthesis and turnover, and their natural functions.

2.2.3.1 Basement membrane and focal adhesion

Basements membranes, also called basal lamina, are sheets of highly organized ECM that are associated with the basal o different type of cells. They establish cell polarity, influence cell metabolism, induce cell differentiation, direct cell migration, and organize membrane receptors. The basement membrane exists as a complex meshwork comprised of the proteins collagen IV, laminin, proteoglycans. The spatial relationships among these molecules were examined is like as “mesh-work. The basements membrane provides an anchor for cells, which adhere to it using specific surface receptors called integrins [6].

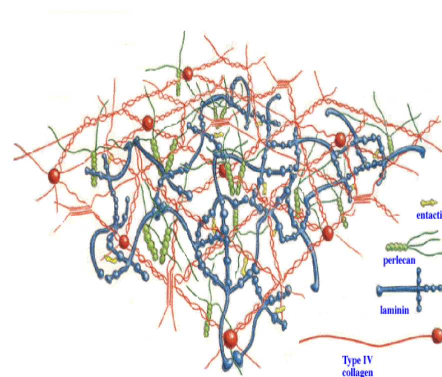


Fig 3 Extracellular matrix structure [Colonna S. et al., 2006]

Focal adhesions are the sites of cell attachment to substrata and then recognized as interfaces with the cytoskeleton [7]. Focal adhesion belongs to the contractile class of

matrix contacts, distinct from protrusive contacts or those that provide mechanical support [8]. The process of anchoring and spreading of cells on substrata occur as multistep processes that involve, among other things, clustering of surface receptors at sites of focal adhesions. Protein complexes link the cytoplasmic tails of surface receptors to the cytoskeleton, facilitating cell adhesion and transmitting signals through the intracellular network that ultimately signal to the nucleus to inform the cell that it has attached. This form of signaling has come to be called "outside in"signalling and is a key component of engineering functional interfaces between materials and living cells [9].

During the attachment of cells to substrata, cells surface receptors recognize ECM components through a specific and reversible process. The initial attraction and attachment often involves nonintegrin adhesion events that recognition the glycosaminoglycans. Once cells adhere, higher affinity interactions such as those involving integrin receptor are stabilized during the spreading phase of adhesion. It is this quality that ensures reversibility of integrin-mediated cell adhesion and allows cells both attach and detach from biological substrata at sites of focal adhesions.

Receptor clustering during adhesion triggers cascades of events involving protein phosphorylation and dephosphorylation that carry intracellular signals through the cell from surface to nucleus, ultimately leading to changes in gene transcription. Changes that occur within cells and that feedback to modulate the activity of surface receptor have come to be called "inside out" signals and modulate activity of both integrin and nonintegrin receptors in focal adhesions.

2.2.3.2 Molecules of ECM

Protein and carbohydrate components of secreted ECM are self-associated through a different type of interactions, including charge properties, ion and metal bridging, hydrophobic domains, redox interactions, and covalent bonding. The structures that form may produce either two-dimensional networks such as the meshwork of the basement membrane, or three-dimensional structures in space such as that of territorial matrix. While most of the assembly is thought to occur extracellularly, there is evidence that

some degree of assembly may be initiated during biosynthesis and take place within intracellular secretory vesicles creating a sort of ”pre-fabricated” scaffold to promote rapid assembly once secretion into the extracellular compartment occurs [10]. In this model system, it has been proposed that distinct intracellular vesicles form and are directionally released during exocytosis; these have been termed “basal laminar vesicles” and “apical vesicles”. The ECM molecules have different roles outside the cells. The collagens form what can be thought of a “functional aggregates” with noncollagenous molecules to form macrostructures including fibrils, basement membranes, filaments, canals, and sheets. The fibronectin is a glycoprotein that binds cell surfaces as well as various other molecules including collagen, heparin sulphate proteoglycans, and fibrin. Fibronectin is involved in different functions including cell migration, wound haling, cell proliferation, blood coagulation, and maintenance of cell cytoskeleton. Fibronectin served as prototype for the development of the RGD-peptides now widely used in modification of biomaterials for the purpose of tissue engineering [11].

Laminin is common ECM component found in basement membranes and used as a substratum for cell migration by many cell types. It has a clear role in cell migration and tissue morphogenesis during embryonic development [12]. It is a favourite ECM-based substrate for cells in the neural system [13].

Proteoglycanes and Glycosaminoglycans play different functions. Perlecan is secreted entirely into the matrix, Syndecan posses a transmembrane domain and remains as an integral component of the plasma membrane, and Glypican is lipid-liked [14].

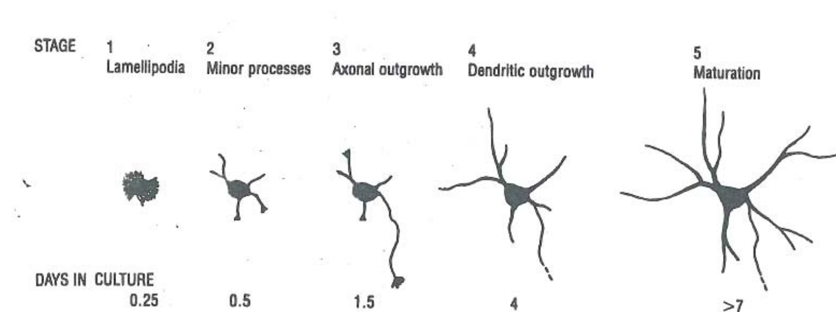
The properties of ECM molecules make them ideal for cell and tissue engineering. Their multifunctional nature makes them ideal for promotion of cell specific adhesion via integrins and other surface receptors. These properties, if well understood, allow the ECM to serve as a rich source for mining and future development of novel tissue engineering applications.

2.2.3.3 Molecules adhesion in neuronal cells

Neurons develop *in vitro* through outgrowth of axon's cone. During this phase of outgrowth, neurons meet numerous microenvironments, some regions contained adhesive molecules and other regions not optimal for the growth. The axon's cone has some receptors (integrines) that they recognize the proteins in the substrate and direct the neuron outgrowth along this path. That can be verified *in vitro*: a portion of nervous tissues in culture on a suitable substrate, it does not extend dendrites and axons on Petri dish; but if the plastic dish comes covered with fibronectin it is possible to observe the increase of dendrites axons extensions. There is an optimal correlation between the outgrowth of axons and the presence of laminin on the substrate, on the contrary, the proteoglycans, inhibits the axonal growth [15]. There is a reason why the glycoprotein laminin have a role in the migration and the outgrowth of the axon. The integrines of the axon's cone have a receptor capable to recognize the sequence RDG and YISRG of the protein laminin and a glycotransferase that recognize particular carbohydrate lateral chains of the molecule of the laminin. [16].

2.2.4 Neurons in culture

Primary hippocampal neuronal cells obtained from 1, 2 days year's old *mesocricetus auratus,* can be plated at low density on plastic Petri dish. By following individual cells plated at low density with time, we can identify characteristic morphological changes that occur in the development of hippocampal neurons in culture. We can divide the events of development of hippocampal neurons in culture into five stages.



In stage 1, shortly after have attached to the substratum, they become surrounded by flattened, motile lamellipodia. In stage 2, with time, the lamellipodia condense at several discrete sites along the cell's circumference; from these sites short, "minor" processes emerge. Minor processes, typically four to six in number, are roughly equal in length (20-30 μm), giving the cells a symmetrical appearance. They are highly dynamic, extending and retracting for short distances over a period of 12 to 24h. Cells at this stage appear to be unpolarized, and cell's processes will become the axon. At stage 3, polarity first, becomes evident when one of the minor processes begins to elongate continuously without retraction until it becomes much longer than the other processes. At this stage, the axon can be distinguished from the other processes on ultrastructural and immunocytochemical grounds. In stage 3 neurons develop a single axon and several minor processes; occasional cells with a multiple axons also are observed. A small number of non neuronal cells also are present. Throughout stage 3, the axon continues to grow at rapid rate, but the remaining minor processes undergo little net elongation. In stage 4, after 2 to 4 days in culture, the remaining minor processes begin to elongate and to acquire the taper and branching pattern characteristic of dendrites. It is at this stage that presynaptic specializations first form on the cell body and dendrites and the microtubule organization typical of mature dendrites first arises.

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. These aspects of development reflect the continuation of processes begun at earlier stages of development. However, in contrast to the events of stages 1 to 4, which appear to be largely endogenously determined, many of these later aspects of neuronal maturation are highly dependent on cell interactions. To emphasize this important difference, we refer to this later period of development as stage 5. Cells in this stage increase the size of cell body and the diameter of dendritic processes that can be identified readily as they emerge from the cell body because of their large diameter, but the thinner, distal portions of dendrites become lost within the neuronal network, as does the

cell's axon. In addition, axons frequently course along the dendrites and, by light microscopy, such axons cannot be distinguished from the underlying dendrites. These features can be resolved by intracellular injection of fluorescent dye such as immunocytochemical markers. The development of immunocytochemical markers that selectively stain axons or dendrites has been a particular important advance for identifying processes in cell culture and for studying their development. 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.

2.3 Polymeric membranes

2.3.1 Introduction

Membranes have gained an important place in tissue engineering and are used in a broad range of applications. The key property that is exploited is the ability of membrane to control the permeation rate of chemical species through the membrane. The membrane may discriminate between the two type's molecules which differ for different size, shape or chemical structure ecc. in the separation process. The basic principle of any separation process is that a certain amount of energy is required to accomplish the separation. Hence, two substances A and B will mix spontaneously when the free enthalpy of the product is smaller than the sum of the free enthalpies of the pure substances. The minimum amount of energy (Wmin), necessary to accomplish complete separation is at least equal to or larger then the free enthalpy of mixing [17]:

$$W_{min} \geq \Delta G_m = \Delta H_m - T\Delta S_m$$

In practice, the energy requirement for separation will be many times greater than this value W_{min}. Different types of separation processes exist and each requires a different amount of energy, but there is a high number of separation process in which membrane

processes can be applied, with a consequently energy saving respect to the traditional methods.

Membrane technology is an emerging technology and because of its multisciplinary character it can be used in a large number of separation processes. The benefits of membrane technology can be summarised as follows:

- separation can be carried out continuously
- energy consumption is generally low
- membrane processes can easily be combined with other separation processes (hybrid processing)
- separation can be carried out under mild conditions
- up-scaling is easy
- membrane properties are variable and can be adjusted
- no additives are required

But drawbacks should be mentioned:

- concentration polarisation/ membrane fouling
- low membrane lifetime
- low selectivity or flux
- up-scaling factor is more or less linear.

Recently news membrane technologies have reduced these disadvantages, creating a very qualitatively process in many applications [18].

2.3.2 Membrane processes

Every membrane separation processes is characterised by the use of membrane to accomplish a particular separation. The membrane has the ability to transport one component more readily then other because of difference in physical and/or chemical properties between the membrane and the permeating components. Transport through the membrane takes place as result of driving force acting on the components in the feed. In many cases the permeation rate through the membrane is proportional to the driving force,

i.e. the flux- force relationship can be described by a linear phenomenological equation. Proportionality between the flux (J) and the driving force is given by

$$J = - A\ dX/\ dx$$

where A is called the phenomenological coefficient and (dX/dx) is the driving force, expressed as the gradient of X (temperature, concentration, pressure) along a coordinate x perpendicular to the transport barrier.

For a pure component permeating through a membrane, it is possible to employ linear relations to describe transport. However, when two or more components permeate simultaneously, such relations cannot be generally employed since coupling phenomena may occur in the fluxes and forces. These coupling phenomena can be described in terms of the formalism of non equilibrium thermodynamics. Other than the driving force, the membrane itself is the principal factor determining the selectivity and flux. In fact the nature of the membrane (its structure and material) determines the type of application, ranging from the separation of microscopic particles to the separation of molecules of an identical size or shape. The product obtained is determined by the applied pressure and the membrane resistance (or permeability).

2.3.3 Membrane properties

Synthetic membranes can be made from a large number of different materials, and can be divided further into organic (polymeric) and inorganic membranes. I would concentrate the attention on the organic membrane, which have been the subject of this study.

Polymers are high molecular weight components built up from a number of basic units, the monomers. The number of structural units linked together to form the long chain molecule is defined as the degree of polymerisation. A polymer chain has an infinite number of different conformations; with increasing of segments the physical, chemical and mechanical properties of the polymer change. When the segment is constituted by a repetition of a single monomer, this polymer is called homopolymer, when the repeating

units are different in the copolymers. In the copolymers, two or plus monomers are coupled together in various ways resulting in a number of different structures.
A polymer used in this study is the Polyester (PE), is a category of polymers which contain the ester functional group in their main chain (Fig.4).
PE membrane is made from a thin, microporous, hydrophilic polyester material film with a high degree of solvent resistance. It is ideal for use in blood assays or general filtration where chemically aggressive solvents may be used.

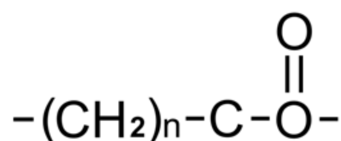


Fig. 4 Chemical structure of Polyester

A polymer used in many applications is the polytetrafluoroethylene (PTFE) (Fig.5). In the 1960's, Dr. Roy Plunkett, a Dupont chemist, was working on a project to find alternate materials for cooling purposes. He had stored some tetrafluorethylene gas (similar to freon) in a container and left it overnight. When he returned in the morning, the gas was gone and in its place was a white wax-like substance. Teflon had been discovered. PTFE is an extraordinary highly inert material, which exhibits excellent thermal and chemical stability. Some say that it is the most useful material known to man. This is because it has a combination of properties that are very hard to find.

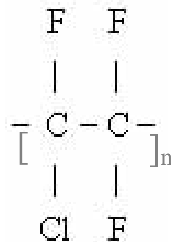


Fig. 5 Chemical structure of Polytetrafluoroethylene

PTFE is composed of carbon and fluorine. Carbon-fluorine and carbon-carbon bonds are among the strongest in single bond organic chemistry. This accounts for many of its properties. Because of the strong bonds, much thermal energy must be used to break down the material.

Because of his hydrophobic nature, is used in a variety of healthcare and industrial applications, as food & beverage, electronics and pharmaceutical industries.

A very important class of polymers are the polysulfones (PSf) and polyethersulfones (PES) (Fig.6).

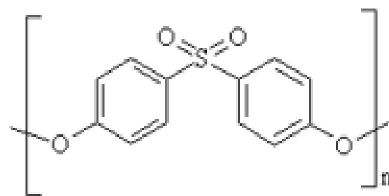


Fig. 6 Chemical structure of Polyethersulfones

The PES posses very good chemical and thermal stability; are widely used as basic materials for ultrafiltration membranes and support materials for composite membranes. Polyethersulfones membranes are also used in the medical sector and in the food sector (membrane technology), because is a low protein binding membrane.

A very important class of polymers are the vinyl polymers, which are obtained by polymerisation of vinyl compounds $H_2C = CHR$, where the side group –R is different for different polymers. The position of the side group R has very important influence on the polymer properties. Between some important vinyl polymers, i would like to quote, in particular the polyacrylonitrile (PAN) with a -CN group, used for made of hollow fiber membrane (Fig.7) [17].

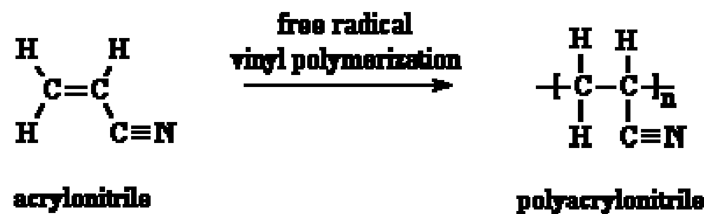


Fig. 7 Chemical structure of Polyacrylonitrile

PAN is a versatile polymeric material commonly used for very few products. In 1893 acrylonitrile was prepared by Moureu, the monomer found use as a copolymer with styrene, and especially as a terpolymer with styrene and butadiene (ABS). Homopolymers of polyacrylonitrile have been uses as fibers and for made asymmetric semipermeable membrane for dialysis and/or ultrafiltration.

The polyetheretherketone (PEEK) is a new chemically and thermally resistant polymer, but with a slow solubility a room temperature. The chemical structure is given in Fig.8.

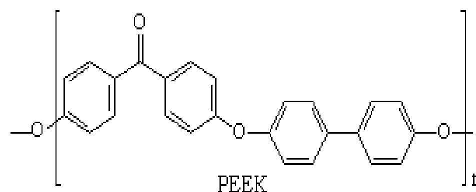


Fig. 8 Chemical structure of Polyetheretherketone

I propose new polymeric membranes made of modified Polyetheretherketone (PEEK-WC) or poly(oxa-1,4-phenylene-oxo-1,4-phenylene-3,3-(isobenzofurane-1,3dihydro-1-oxo)-diyl-1,4-phenylene) as materials with potential for use for cell culture in biohybrid systems(Fig.9). Membranes were prepared from PEEK-WC, which exhibits chemical stability and excellent thermal and mechanical resistance similarly to traditional PEEKs, which are used in medical implants. Differently from PEEKs, PEEK-WC is soluble in various solvents owing to lack of crystallinity. This characteristic allows its use for preparing membranes by phase inversion, which is cheap and flexible method. Previous studies have shown that this novel membrane is very promising for various applications [19]. The developed PEEK-WC membranes combine the advantageous properties of the polymer with those of membranes such as permeability, selectivity and stability.

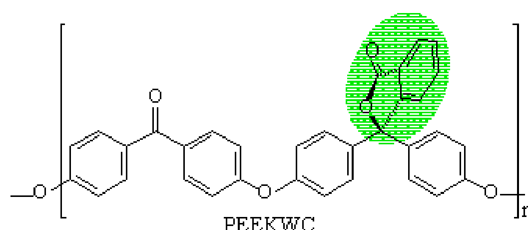


Fig. 9 Chemical structure of modified polyetheretherketone

2.3.4. Membrane preparation

A number of preparation techniques exist for preparing a membrane to be constructed from a given polymer. The kind of technique employed depends mainly on the material used and on the desired membrane structure. Three basic types of membrane can be distinguished based on structure and separation principles:

- Porous membranes (microfiltration, ultrafiltration)
- Nonporous membranes (gas séparation, pervaporation, dialysis)
- Carrier membrane

In this thesis microporous and dense membranes have been used.

Membranes induce separation by discriminating between particle size. Such membranes are used in microfiltration and ultrafiltration. High selectivities can be obtained when the solute size is large relative to the pore size in the membrane.

Porous and dense membranes are obtained by phase inversion. This is a very versatile technique allowing all kind of morphologies to be obtained.

Phase inversion is a process whereby a polymer is transformed in a controlled manner from a 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 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. The concept of phase inversion covers a range of different techniques such as solvent evaporation, precipitation by controlled evaporation, thermal precipitation, precipitation from the vapour phase and immersion precipitation. The majority of the phase inversion 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 nonsolvent. The membrane structure ultimately obtained results from a combination of mass transfer and phase separation.

"Phase inversion" refers to the controlled transformation of cast polymer film from a liquid solid state. During this process, a thermodynamically stable polymer solution is mostly subject to a controlled liquid-liquid demixing during which the cast polymer film "phase separates" into a polymer –rich and polymer-lean phase, ultimately forming the matrix and the pores of the membrane, respectively. The thermodynamic behaviour of polymer solution subjected to immersion-precipitation can be represented in a polymer/solvent/non-solventphase diagram (Fig.11).

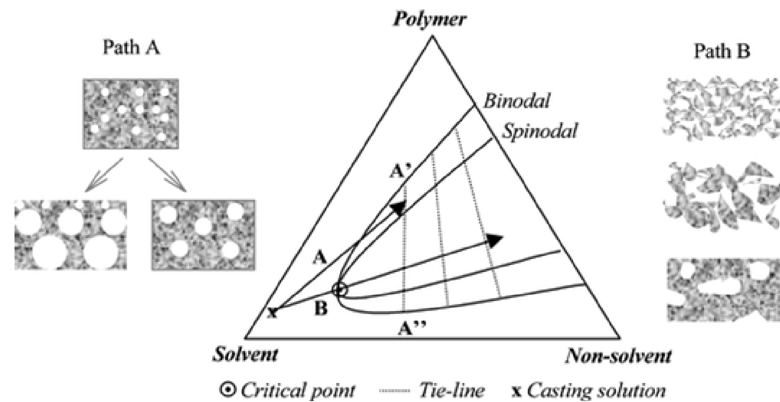


Fig. 11 Schematic representation of the mechanism of phase separation during membrane formation

In this diagram, the initial polymer solution is situated in the stable region outside the bimodal. After "binodal demixing", indicated by pathway A, which is the most common phase separation mechanism, polymer solutions arrive in the metastable region between the binodal an the spinodal. In this region, polymer solutions will "phase-separate" into a polymer-lean and a polymer-rich phase, indicated on the phase diagram by A' and A'' tieline ends respectively. Phase-separation takes place according to the nucleation and growth mechanism, in which the formed nuclei grow and most often progress towards a phase-coalescence. "Spinodal decomposition", the second and less frequent mechanism is represented by pathway B. This occurs whenever the polymer solution directly moves to the thermodynamically unstable zone within the spinodal. Again, two different phases are formed, but instead of developing well-defined nuclei, two co-continuous phases will be formed.

However, not only the thermodynamic but also the kinetic aspect of phase inversion process should be considered, more specifically the moment at which the developing membrane structure gets solidified.

Membranes in flat and tubular configuration can be prepared by immersion precipitation. Flat membranes are used in plate-and-frame and spiral-wound systems whereas tubular membranes are used in hollow fiber, capillary and tubular systems.

For preparation of flat membrane, the polymer is dissolved in a suitable solvent or solvent mixture (which may include additives). The viscosity of the solution depends on the molecular weight of the polymer, its concentration, the kind of solvent (mixture) and the various additives. The casting thickness can vary roughly from 50-500 µm. The cast film is then immersed in a nonsolvent bath where exchange occurs between the solvent and nonsolvent and eventually the polymer precipitates. Water is often used as nonsolvent but organic solvents can be used as well. Since the solvent/nonsolvent pair is a very important parameter in obtaining the desired structure (Fig.9). Other preparation parameters are: polymer concentration, evaporation time, humidity, temperature, and the composition of casting solution (e.g. additives). These parameters are mainly determining the ultimate membrane performance (flux and selectivity) and hence for its application.

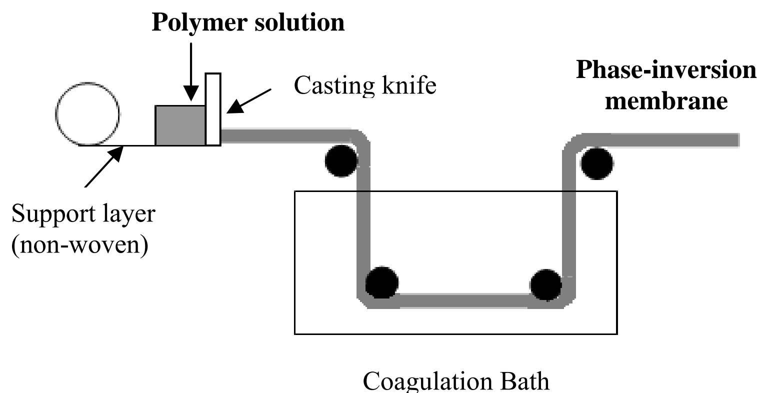


Fig. 9 Schematic representation of flat membrane preparation

The tubular form is alternative geometry for a membrane. On basis of differences in dimensions, the following types may distinguish:

a) hollow fiber membranes (diameter: < 0.5 mm)

b) capillary membranes (diameter: 0.5-5 m)
c) tubular membranes (diameter: > 5 m)

The dimensions of the tubular membranes are so large that they have to be supported whereas the hollow fibers and capillaries are self-supporting. Hollow fibers and capillaries can be prepared via three different methods:

- wet spinning (or dry-wet spinning)
- melt spinning
- dry spinning

In particular, i would to talk of the dry-wet spinning process, because used in this thesis for preparation of hollow fiber membrane.

A viscous polymer solution containing a polymer, solvent and sometimes additives is pumped through a spinneret, the polymer solution being filtered before it enters the spinneret. The bore injection fluid is pumped through the inner tube of the spinneret. After a short residence time in the air or a controlled atmosphere (the term dry originates from this step) the fiber is immersed in a nonsolvent bath where coagulation occurs. The fiber is then collected upon a godet (Fig.10).

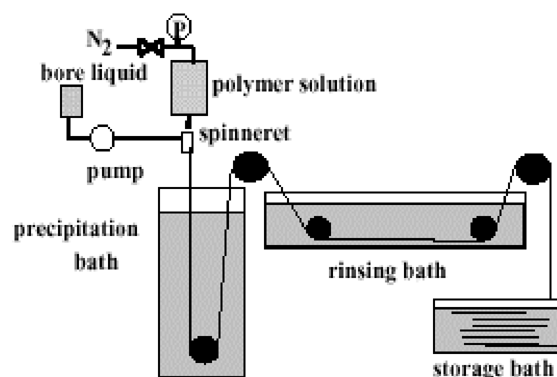


Fig. 10 Schematic representation of spinning apparatus

The main spinning parameters are: the extrusion rate of polymer solution; the bore fluid rate; the tearing-rate; the residence time in the air gap; the dimensions of the spinneret. These parameters interfere with the membrane forming parameters such as the composition of polymer solution, the composition of the coagulation bath, and its temperature [17].

A wide variety of experimental parameters have been shown to have an impact on the final morphology of asymmetric membranes, and consequently on their performance [20].

2.3.5. Characterization of membrane

Membranes need to be characterised to ascertain which may be used for a certain separation or class of separations. A small change in one of the membrane formation parameters can change the top layer structure and consequently have a drastic effect on membrane performance. Membrane characterisation is necessary to relate structural membrane properties such as cut off, pore size, pore size distribution, free volume, ecc. The information obtained from characterisation measurements will help us in the prediction of membrane performance for a given application.

Characterisation data for porous membranes often give rise to misunderstandings and misinterpretations. It should be realised that even when the pores sizes and pore size distributions have been determined. However, in actual separation processes the membrane performance is mainly controlled by other factors, e.g. concentration polarisation and fouling.

In general, the pores in these membranes do not have the same size but exist a distribution of pore sizes. The membrane can be characterised by a nominal or an absolute pore size. With an absolute rating, every particle or molecule of that size or larger is retained. On the other hand, a nominal rating indicates that a percentage (95-98%) of the particles or molecules of that size or larger is retained.

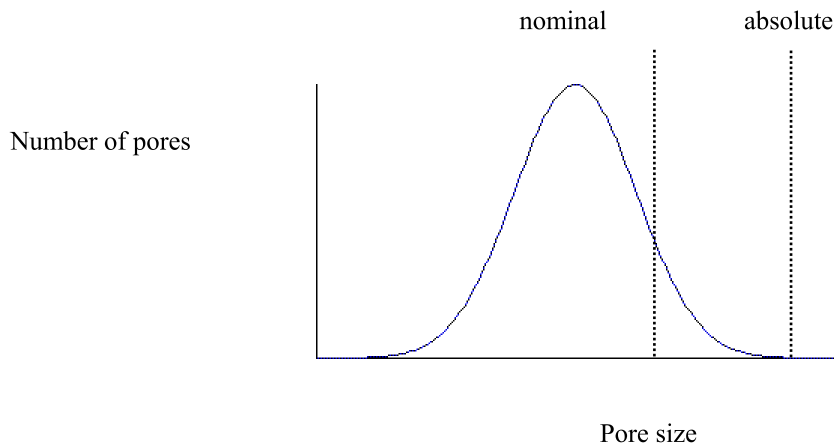


Fig. 12 Schematic drawing of the pore size distribution in a certain membrane

Another factor of interest is the surface porosity. This is also a very important variable in determining the flux through the membrane, in combination with the thickness of the top layer or the length of the pore.

Two different types of characterisation method for porous membranes can be distinguished from the above considerations:

- Structure –related parameters: determination of pore size, pore size distribution, top layer thickness and surface porosity
- Permeation related parameters using solutes that are more or less retained by membrane (cut-off measurements).

The microfiltration membranes (which used in this thesis) are readily characterised with various techniques:

- scanning electron microscopy
- atomic force microscopy
- permeation measurements

The first thwo methods listed involve the measurement of morphological or structural-related parameters whereas the last method is typical permeation-related technique.

2.3.5.1 Scanning electron microscopy

The scanning electron microscopy (SEM) is a very simple method for characterising and investigating the porous structure of microfiltration membranes, especially for asymmetric membrane. The resolution limit is in the 10 nm range, whereas the pore diameters of microfiltration membranes are in the 0.1 to 10 μm range.

The principle of the scanning electron microscope is: the incident electrons are called primary (high energy electron) electrons, are reflected producing secondary electrons. These secondary electrons (low energy) determine the imaging. Scanning electron microscopy allows a clear view of the overall structure of a microfiltration membrane; the top, the cross-section and the bottom surface can all be observed very nicely. Micrographs of this kind allow the pore size, the pore size distribution and the surface porosity to be obtained. Also the geometry of the pores can be clearly visualised.

2.3.5.2 Atomic force microscopy

Atomic force microscopy is rather new method to characterise the surface of a membrane. A sharp tip with a diameter smaller then 100 Å is canning across a surface with a constant force. London-vander Waals interactions will occur between the atoms in the tip and the surface of the sample and these forces are detected. This will result in a line scan or profile of the surface. The use of a micro fabricated cantilever allows to operate at very low forces, makes possible to apply this technique for soft surface as in polymeric membrane.

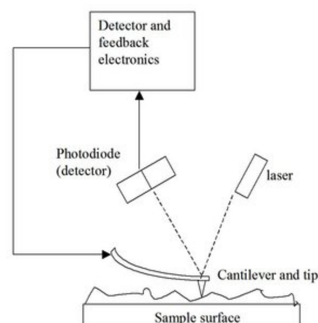
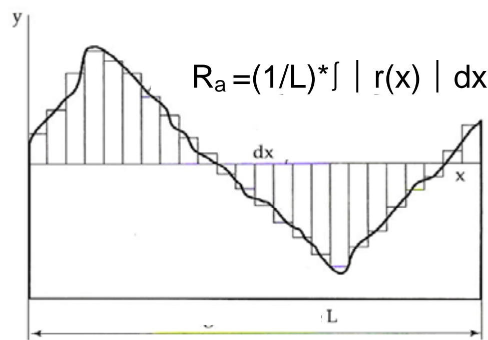


Fig. 12 Schematic drawing of the atomic force microscopy

The obtained line scans do not only reveal the possible position and size of pore, also an indication of surface roughness is obtained.

The average roughness (Ra) is the commonly used roughness parameter; Ra is an integral of the absolute value of roughness profile. It is the shaded area divided by the evaluation length.



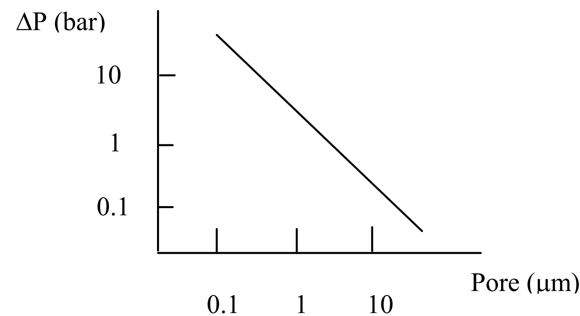
2.3.5.3 Permeability method

If capillary pores are assumed to be present, the pore size can be obtained by measuring the flux through a membrane at a constant pressure using the Hagen-Poiseuille equation:

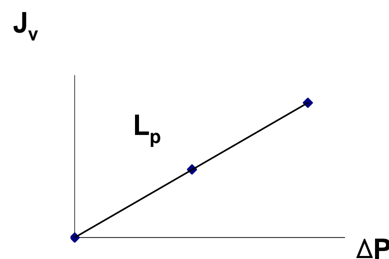
$$J = (\varepsilon\, r^2 / 8\, \eta\, \tau) * (\Delta P / \Delta x)$$

Where J is the (water) flux through the membrane at a driving force of $\Delta P/\Delta x$, with ΔP being the pressure difference and Δx the membrane thickness. The proportionality factor contains the pore radius r, the liquid viscosity η, the surface porosity of the membrane ε and the tortuosity factor τ.

The method itself is very simple, the (water) flux through the membrane is measured as function of the applied pressure. At a certain minimum pressure the largest pores become permeable, while the smaller pores still remain impermeable.



This minimum pressure depends mainly on the type of membrane material present (contact angle), type of permeant (surface tension) and pore size. The increase in (water) flux is proportional to the increase in applied pressure. Very small pore diameters require a high pressure to wet the membrane. At certain pressure, the membrane becomes wetted an permeable, and thereafter the flux increases linearly with increasing pressure.



The Hagen-Poiseuille relationship assumes that the pores in the membrane are cylindrical but generally this is not the case. Therefore, these limitations should be considered carefully in applying this equation.

The Kozen-Carman equation can be used instead of the Hagen-Poiseuille equation. It is assumed in this equation that the pores are interstices close-packed spheres as can be found in sintered structures. The flux is given by this equation:

$$J = (\varepsilon^3 / K\, \eta\, S\, (1-\varepsilon)^2) * (\Delta P / \Delta x)$$

Where K is a membrane constant, called the Kozeny-carman constant, which is dependent on the pore shape and tortuosity. Here, ε is the porosity and S is the specific surface area [17].

2.4 Cell interaction with polymers

Synthetic polymers are an importantsw element in new strategies for producing engineered tissue. Several classes of polymer have proved to be most useful in biomedical applications. But to select appropriate polymers for tissue engineering, it is necessary to understand the influence of the polymer on cell viability, growth, and function.

Cell interactions with polymers are usually studied using cell culture techniques. To study cell interactions, cells in culture are plated over a polymer surface and extent of cell adhesion and spreading on the surface is measured. By maintaining the culture for longer periods, the influence of the substrate on cell viability, function and motility can also be determined.

Most tissue-derived cells are anchorage dependent and require attachment to a solid surface for viability and growth. For this reason, the initial events that occur when a cell approaches a surface are fundamental interest. In tissue engineering, cell adhesion to surface is critical because adhesion precedes other events, such as cell spreading, cell migration, and, often, differentiated cell function. A number of different techniques for quantifying the cell adhesion have been developed. In this thesis is used a simple fluorescent methods for quantifying the cell adhesion to surface and the number of attached cells can be determined by direct visualization.

Cells adherent form aggregates, permitting correlation of cell-cell interactions with cell differentiation, viability, and migration, as well as subsequent tissue formation.

Cells function in the culture consequently must be monitored; for example, protein secretion and detoxification, metabolic and catabolic activity of cells during the changes in the morphology in the culture [21].

2.4.1 Effect of the polymer chemistry on cell behaviour

For cells attached to a solid substrate, cell behaviour and function depend on the characteristics of the substrate. A number of groups have examined the relationship between chemical or physical characteristics of the substrate and behaviour or function of attached cells. Cell adhesion appears to be maximized on surfaces with intermediate wettability [22]. The effect of surface wettability is investigated from Lee et al., on polyethylene (PE) surfaces. Induction and growth of neurites from rat pheachromocytoma (PC-12) cells attached on the polymer surfaces with different hydrophilicity were investigated using the wettability gradient polyethylene (PE) surfaces. It was observed that neurite formation of PC-12 cells was increased more onto the position with a moderate hydrophilicity of the wettability gradient surface then onto the more hydrophobic or hydrophilic positions. Surface wettability plays an important role for neurite formation on polymer surfaces for axon regeneration.

For most surfaces, adhesion requires the presence of serum and, therefore, this optimum is probably related to the ability of proteins, such as fibronectin, to absorb to the surface. In the absence of serum, adhesion is enhanced on positively charged surface.

Cell viability may also be related to interactions with the surface. Polymers can frequently be made more suitable for cell attachment and growth by surface modification, such surface modification with proteins such as fibronectin a vitronectin. On the other hand, some reports have identified specific chemical groups at the polymer surface, such (-OH) or C-O functionalities as important factors in modulating the fate of surface-attached cells [23]. So far, no general principles that would allow prediction of extent of the extent of attachment, spreading, or growth of cultured cells on different polymer surfaces have been identified. Parameters such, density of surface hydroxyl groups, surface free energy, protein adsorption, water content are very important for cell attachment [24].

2.4.2 Biodegradable polymers

Biodegradable polymers slowly degrade, this feature may be important for many tissue engineering applications, because the polymer will disappear as functional tissue

regenerates. Biodegradable polymers may provide an additional level of control over cell interactions: during polymer degradation, the surface of the polymer is constantly renewed, providing a dynamic substrate for cell attachment and growth.

Homopolymers and copolymers of poly(L-lactic acid), poly(glycolic acid), and poly(lactide-co-glycolide) have been frequently examined as cell culture substrates, because they have been used as implanted sutures for several decades [25].

Poly(D,L-lactic acid) macroporous guidance scaffolds (foams) for neuronal survival, axonal regeneration were studied [26]. The foams were prepared by thermally induced polymer-solvent phase separation process and contained longitudinally oriented macropores connected to each other by a network of micropores. Cells from spinal cord regenerate axons on these foams in a similar *in vivo* structure.

2.4.3 Synthetic polymers with adsorbed proteins

Cell attachment, migration and growth on polymeric surfaces appear to be mediated by proteins, either adsorbed from the culture medium or secreted by the cultured cells. Because it is difficult to study these effects in situ during cell culture, often the polymer surfaces are pretreated with purified protein solutions (conditioning). Cell spreading, but not attachment has been correlated with fibronectin adsorption to a variety of surfaces. Rates of cell migration on a polymeric surface have been shown to depend on the concentration of preadsorbed adhesive proteins, as well as the presence of soluble inhibitors to protein-mediated cell adhesion [27].

Poly-D-lysine, poly-L-lysine, collagen, laminin, fibronectin, and Matrigel were compared with standard tissue grade polystyrene for their impact on the expansion and neuronal differentiation of mesenchymal stem cells (MSCs), in the work of Qian et al.[28].

2.4.4 Hybrid polymers with immobilized functional groups

Surface modifications techniques have been used to produce polymers for cell attachment. For example, chemical groups can be added to change the wettability of the surface, which often influences cell adhesion as described above. Alternatively, whole proteins such as

collagen can be immobilized to the surface, providing the cell with a substrate that more such as collagen can be immobilized to the surface, providing the cell with a substrate that more closely resembles the ECM found in tissues. Smaller active functional groups have been used to modify surfaces such oligopeptides, saccharides [29] or glycolipids. Certain short amino acid sequences appear to bind to receptors on cell surfaces and mediate cell adhesion. For example, the cell-binding domain of fibronectin contains the tripeptide RGD (Arg-Gly-Asp). Cells attach to surfaces containing adsorbed oligopeptides with RGD sequence and soluble, demonstrating the importance of this sequence in adhesion of cultured cells [30].

Poly (ethylene-covinyl alchol) (EVAL) membranes were modified by covalent bonding of lysine via isocyanation of surface hydroxyl groups to improve cell behaviour in cultured cerebellar granule neurons from 7-day-ol Wistar rats, in the study of Young et al. (Tab.2). Neurons seeded on the lysine-immobilized EVAL membrane were able to regenerate with the formation of an extensive neurotic network, indicating that the surface modification on the EVAL membrane should be useful for culturing of neurons [31].

Lakard et al. present as an alternative to poly-L-lysine, new polymer film substrates, realized by electro-polymerisation of different monomers on fluorine-doped tin oxide (FTO) surfaces since electro-polymerisation is a good method to coat selectively metallic or semiconducting electrodes with polymer films. So, the adhesion, proliferation and morphology of rat neuronal cell lines were investigated on polymer treated surfaces. Several amine-based polyehyleneimine (PEI), polypropyleneimine (PPI), polylpirrole (PPy) and poly(p-phenylenediamine) (PPD). These polymer films are coated on FTO surfaces by electrochemical oxidation. Cells adhesion and proliferation are tested after 72 h of culture, The best results are obtained on PEI and PPI, we deduce that polymers can be useful as coating surface to cultivate neuronal cells [32].

Laminin-derived cell-adhesive peptides were coupled to Poly(tetrafluorethylene-co-hexafluoropropylene) (FEP) film surfaces functional groups, are propose from Tong et al. (Tab.1) for analyze neurite outgrowth (number and length of neurites), cell adhesion and

viability. This study has demonstrated that neurite outgrowth and adhesion were significantly better on peptide-modified surfaces than on FEP [33].

2.4.5 Influence of surface morphology on cell behaviour

The behaviour of cultured cells on surfaces with edges, grooves or other textures is different than smooth surfaces. In many cases, cells oriented and migrated along fibers or ridges in the surface, a phenomenon called contact guidance from early studies on neuronal cell cultures [34]. In the *in vitro* study of McKenzie et al. the cytocompatibility properties of formulations containing carbon nanofibers pertinent to neural implant applications were determinate. Substrates were prepared from four different types of carbon fibers, two with nanoscale diameters (nanophase, or less than or equal to 100 nm) and two with conventional diameters (or greater than 100 nm). Astrocytes were seeded onto the substrates for adhesion, proliferation, and long-term function studies. Results provided the first evidence that astrocytes preferentially adhered and proliferated on carbon fibers that had the largest diameter. The decrease of astrocytes on carbon nanofiber with nanoscale diameter may use for positive interactions with neuronal cells [35]. Substrates with peaks and valleys also influenced the function of attached cells.

2.4.6 Use of patterned surfaces to control cell behaviour

A variety of techniques have been used to create chemically patterned surfaces containing cell adhesive and non adhesive regions. Micropatterned surfaces are also used in cell culture. For example Polymethilsiloxane (PDMS) surfaces with 10- to 50- μm wide are used for maximized the Schwann cell attachment. The smaller pattern widths increase the degree of orientation and regardless of interval width. Elastomeric stumps of PDMS may enhance peripheral nerve regeneration by creating a highly ordered Schwann cell matrix for guidance of neurons [36].

PDMS microstamps were used to create poly-d-lysine (PDL) substrates permissive to cell attachment and growth, and polyethylene glycol (PEG) substrates were used to minimize protein and cell adhesion in the work of Branch et al. Hippocampal cells plated on uniform PEG substrates gave a steady increase in biofilm thickness on PEG films

throughout the culture, possibly from proteins of neuronal origin. The authors found that all the layers in the cross-linking procedure were stable in cell culture conditions, with the exception of PEG, possibly from proteins of neuronal origin [37].

Vogt et al. (Tab.2) demonstrated that rat embryonic cortical neurons can be grown on patterns of extracellular matrix proteins applied to polystyrene substrates by microcontact printing. Cells comply well with the pattern and form synaptic connections along the experimentally defined pathways. Chemical synapses identified by double patch-clamp measurement showed paired pulse depression as well as frequency-dependent depression in response to trains of stimuli. This type of short-term plasticity has similarly been reported by others in brain slices. Thus, the system reproduces features central for neuronal information processing while the architecture of the network is experimentally manipulable. The ability to tailor the geometry of functional neuronal networks offers a valuable tool both for fundamental questions in neuroscientific research and a wide range of biotechnological applications [38].

Song et al. describe a method for preparing substrates with micropatterns of positive guidance cues for the purpose of stimulating the growth of neurons. This method uses a oxidizing potential, applied to a micropatterned of indium tin oxide in the presence of pyrrole and polyglutamic acid, to electro-deposition a matrix consisting of polypyrrole doped with polyglutamic acid the resulting matrix subsequently can be modified with positive guidance cues via standard amide coupling reactions. Cells adhered to positive guidance cues and neurite extensions were found to occur almost exclusively in areas where positive guidance cues [39].

2.4.7 Cell interactions with three-dimensional polymer scaffolds and gels

Cells within tissues encounter a complex chemical and physical environment that is quite from commonly used cell culture conditions. Many investigators recently use three-dimensional .cell culture method to simulate the chemical and physical environment of tissue [40].

Gel agarose has been used for three-dimensional cell culture. Neurites produced by PC-12 cells within agarose gels, even under optimal conditions, are much shorter and fewer in number then neuritis produced in gels composed of ECM molecules however [41].

2.5 Applications: membrane bioreactor

In vitro tissue engineering is constitute in three components cells, scaffold or materials and signalling system, which even are the basic components of biologic tissues. *In vitro* bioreactor can be used for controlled studies of tissue growth and function. Cells isolated by enzymatic digestion, inoculated in bioreactor containing 3D polymer scaffolds, can be expanded in monolayer. Structural (cells adhesion, cell number, ECM developing, differentiation and growth), and functional (cell metabolism: glucose consumption and lactate production, proteins synthesis) assessment of tissue engineering can be analyzed in a dynamic system as a bioreactor [42].

Differently from static culture methods the bioreactors allow the culture of cells under tissue specific mechanical forces such as pressure, shear stress and interstitial flow. Furthermore, in this system a constant turnover of tissue culture medium augments the gas and nutrient exchange, which together with the complete fluid dynamics control ensures the long-term maintenance of cell viability and functions [43].

One of the most-used bioreactors for mammalian tissue growth is the hollow fiber membrane bioreactor. This bioreactor meets the main requirements for cell culture: wide area for exchange of oxygen/carbon dioxide and nutrient transfer, removal of catabolites and protection from shear stress. To date hollow fiber membrane bioreactor has mainly been used for large-scale mammalian cell culture to produce products as monoclonal antibodies, for expansion of tumor-infiltrating T lymphocytes. Although the hollow fiber bioreactor is used in the cell culture improvements in the materials and design are desirable to ensure the maintenance of in vitro human lymphocyte model system. De Bartolo et al. in this work report on modified polyetheretherketone hollow fiber (PEEK-WC-HF) membrane bioreactor as potential system to be used for lymphocyte culture [44].

Galactosylated polyethersulphone (PES) membrane bioreactor were proposed from De

Bartolo et al. as enables the long-term maintenance of liver-specific functions of human hepatocytes under continuous perfusion. Human hepatocytes cultured in the membrane bioreactor under continuous perfusion for 21 days, were maintained their liver-specific functions for the whole culture time in terms of urea synthesis and albumin production as well as protein secretion.

This study demonstrated that the galactosylated membrane bioreactor is able to support extended *in vitro* hepatocyte functions [45].

An oxygen-permeable membrane bioreactor utilizing human hepatocytes has been tested from De Bartolo et al. In the bioreactor, human hepatocytes were cultured between flat-sheet gas-permeable polymeric membranes, which ensure the diffusion of O_2 and CO_2 providing a support for cell anchorage and growth and permit the online observation of the cells with an inverse microscope. This bioreactor allows a direct oxygenation of cells adhered on membranes and of the medium overlaying cells simulating in vivo sinusoidal organization. Human hepatocytes were cultured in the presence of some therapeutic molecules to assess the temporal liver-specific functions of the cells.

The ability of hepatocytes to perform liver-specific functions in terms of urea and albumin synthesis, as well as secretion of total proteins, was maintained for 32 days.

This study attested the feasibility of the membrane bioreactor as an in vitro simple model system that allows human hepatocytes to be maintained in a differentiated state similar to that in vivo [46].

Sertoli cells differentiate into dopaminergic NT2N neurons are cultured in the high aspect ratio vessel (HARV) rotating wall bioreactor. SC-NT2 tissue construct may be an important source of dopaminergic neurons for neuronal transplantation [47].

In following two tables (Tab.1-2) are reported the principal papers postings in the last years on neuronal cells and biomaterials. In these are synthesised the important parameters which have characterised the work (Substrate, Morphological and Chemical properties, Cell type).

References	Substrate	Cell Type	Morphological and Chemical Characteristics
M.Matsuzawa, BioSystem, 1995	Coverslip chemically modified with a self-assembled monolayer film of n-octadecyltrichlorosilane OTS, covalently blonded with n-(2-aminoethyl-3-aminopropyl)trimethoxysilane EDA or with trimethoxysilydiethylenetriamine DETA.	SK-N-SH human neuroblastoma cells; embryonic rat hippocampal neurons	Chemically patterned substrates with amino groups
M.Matsuzawa J.of NeuroSc. Methods, 1996	Amine-derived glass surface coated with a synthetic peptide P20 (derived from a neurite –outgrowth-promoting domain of the B2 chain of mouse laminin)	Rat hippocampal neurons	Amine derivated surface
M.Scholl J.of NeuroSc. Methods, 2000	Adhesion peptide PA22-2 (derivated from laminin A-chain) applied onto silicon oxide surfaces by microcontact printing using a PMDS (polydimethylsiloxane) stamp.	Embrionic Rat hippocampal neurons	Immobilitation of biomolecules
Y.W.Tong, Biomat., 2001	Laminin-derived cell adhesive peptides (YIGSR and IKVAV) coupled to FEP (polytetrafluoroethylene-co-hexafluoropropylene); FEP-[N\O] (FEP aminated)	Embryonic rat hippocampal neurons	Peptide-modified surface
M.E. Manwaring, Biomat., 2001	CA (cellulose acetate), PAN-PVC (polyacrylonitrile-polyvinyl chloride), PES (polyether sulfone), PEVAC (polyethylene vinyl acetate), TECO (Tecoflex) PP (polypropylene),with laminin coating	Rat meningial and glial cells	Different hydrophobic membrane
P.Heiduschka, Electroch.Acta, 2001	Polylysine and laminin coated silicon microstructures	Adult rat retina neurons	Surface electrochemically modified
S.M.Potter J.of NeuroSc. Methods, 2001	FEP (fluorinated ethylene-propylene) and FC	Rat cortical neurons	A transparent hydrophobic membrane Selectively permeable to oxygen and carbon dioxide
S.J.Lee, J.of Colloid and Interfase Sc., 2003	PE (polyethylene)	PC-12 cell line	Hydrophilic membrane
T.H.Young, Biomat., 2003	EVAL with covalent bonding of lysine	Cerebellar Granule Neurons	Dense membrane
S.Lakard, Bioelectroch., 2004	PEI (polyethyleneimine), PPI (polypropyleneimine), PPy (polypyrrole) and PPPD poly(p-phenylenediamine)-coated FTO (fluorine-doped tin oxide) surfaces.	Rat neuronal cell lines (13S124)	Amino polymers
Lichuan Qian, Biomat., 2004	Poly-D-lysine, poly-L-lysine, collagen, laminin, fibronectin,and Matrigel coated polystyrene dishes	Human mesenchymal stem cells (MSCs)	Natural substrate
J.L.mcKenzie, Biomat., 2004	(PCU:CN) Polycarbonate urethane to Carbon nanofiber.	Rat astrocytes	Surface with high nanometer roughness
S.Saporta, Brain Research Bulletin, 2004	High aspect ratio vessel (HARV)-Matrigel	Sertoli cells, Cells line (NT2)	Rotating all bioreactor

Tab.1

References	Substrate	Cell Type	Morphological and Chemical Characteristics
C.M.Patist, Biomat., 2004	Poly(D,L-lactic acid) macroporous scaffolds(foams)	Rat thoracic spinal cord	Scaffolds for implantation
A.K. Vogt Neuroscience., 2005	Surface patterning -PDMS (polydimethilsiloxane) with poly-D-lysine	Rat cortical neurons	Surface micropattern with extracellular matrix molecules
N.Zhang, Brain Research Review, 2005	Different Biomaterials (Scaffolds, Bridge, HFMs)	Spinal Cord	Different configuration of biodegradable materials (PGA, PLA, PLGA)
K.E.Schmalenber Biomat., 2005	PMMA poly(methylmethacrylate)+[lmn]	Schauwn cells	Hydrophilic surface
T-H. Young Biomat., 2005	PVA (polyninylalcohol) and EVAL (polyethylene-*co*-vinylalcohol) substrates	Embrionic rat cortical stem cells	EVAL contains both hydrophilic vinyl alcohol segments and hydrophobic ethylene segment; PVA
D.A.Heller Biomat., 2005	PDMS (polydimethilsiloxane) stamps "inked" with PA22-2 peptide and transferred onto gold coverslips	Mouse hippocampal neurons	Well-ordered patterns of the peptide on gold. Roughness of ~1 nm
J.H.Wang Biomat., 2006	LAS (lysine-alanine sequential polymer substrates at neurosphere level)	Embryonic rat cerebral cortical stem cell	Heterologous polymer able to enhance axon growth in a serum-free medium
R. William Biomat., 2006	PPy(Polypyrrole) +laminin fragments (CDPGYIGSR-p31 and RNIAEIIKDI-p20) coated plastic coverslips	Rat cortices cells E18	Electrical conductivity
H.K.Song, Biomat., 2006	pPY + [Pglu] [PLys] pPY + [Pglu] [Lmn]	Dorsal root ganglia	Anionic surface
I.A.Kim J.of Bioscience and Bioengineering, 2006	Microfiber-patterned substrates (PLGA-poly-D,L-lactide-co-glycolic acid dissolved in 1:1 mixture of DMF (N,N-dimethylformamide and THF(tetrahydrofurane) depositated on laminin-coated coverglass	Rat PC-12 cells	Fibrous substrates (fiber with diameters from 3 to 5 µm to serve as a guide for cell outgrowth
E.Schnell Biomat., 2007	Aligned (PCL) poly-εcaprolactone and collagen/PCL (C/PCL) nanofibers-coated coverslips	Rat and chick dorsal root ganglia, Schwann, Fibroblasts and Olfactory ensheathing cells	Adhesive capacity of electrospun nanofibers in combination with a non- adhesive substrate, synthetic or natural polimers
N.Gomez Biomat., 2007	NGF immobilized on PDMS stamps with PAA-azido and microchannels	Rat hippocampal neurons	Homogeneously immobilitation of NGF;

Tab.2

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LIST OF SYMBOLS

A= membrane surface (m^2)

r = (pore) radius (m)

S_m = Entropy of mixing (J/mole.K)

G_m= Free energy of mixing (J/mole)

H_m = Enthalpy of mixing (J/mole)

K = kozeiny-Carman constant (-)

P = hydrodynamic pressure, M L^{-1} T^{-2}

S = Surface area (m^2 /g)

X = driving force (N/mole)

T = temperature (K)

Greek symbol

τ = tortuosity (-)

ε = porosity (-)

η = viscosity (Pa.s)

γ = surface tension

Chapter 3

Chapter 3

Membrane bio-hybrid system in neuro-biotechnology

3.1. Abstract

At present a great deal of attention is being directed towards the possibility of replacing or restoring the anatomical structural organization of damaged neuronal regions and consequently their functional capacities by developing artificial systems based on biomaterials, scaffolds and cells. Among the biomaterials applied in the field of neuro-biotechnology, polymeric semi-permeable membranes could provide the mechanical support required for the regulation of cell growth in bio-hybrid systems. The ultimate goal of this technique might be very well achieved by appropriate bio-interactions of desired cell responses (i.e. *in vitro* induction of a neural circuit, *in vivo* fabrication of a tissue structure). The great impact of membrane bio-hybrid systems in tissue engineering has allowed the designing of a physiological model such as neuronal elements. These may turn out to be a key approach for studying pathogenic states as well as, and more important, the development of appropriate bio-molecules for therapeutic purposes. In view of the widespread structural organization of all the brain regions, it is the aim of this study to focus our attention on the potential value of this biotechnological approach to a functionally key region such as the hippocampus. Indeed, the principal neurons of this brain region, i.e. pyramidal cells, are actively involved in many hippocampal-dependent neurophysiological functions, such as memory and learning. This makes them a valuable tool to investigate not only their synaptic plasticity properties, but also neurodegenerative events through the distribution and quantification of microtubule-associated protein type 2. In this work, the reconstruction of membrane bio-hybrid systems, constituted of isolated cells and membranes, appears to represent a crucial step for the success of these systems. Moreover, the optimization of transport, physico-chemical and structural properties of the membrane as well as fluid dynamics of cellular microenvironments tend to favor cell-membrane interactions and the functional maintenance of hippocampal cells. As a consequence the feasibility of developing a hippocampal cell membrane bio-hybrid system capable of regenerating a neuronal network

could prove to be an important approach for studying the behavior of neuronal populations in some of most common neurodegenerative disease such as Alzheimer's disease.

3.2. Introduction

With the purpose of simulating *in vitro* biological phenomena, scientists have begun to construct artificial membranes that may be handled in both industrial and medical applications. Artificial lungs (blood oxygenation) and kidneys (hemodialysis) are just the two oldest examples. The use of polymeric semi-permeable membranes with different physico-chemical and transport properties is an appealing approach in the tissue and bio-artificial organ engineering field, since these bio-membranes share specific features such as the selective transport of molecules, resistance and protection. Furthermore, synthetic membranes are easily and readily produced in copious amounts so that their morphological and physico-chemical properties can be modulated for numerous and specific applications.

Ever since 1933, when Vincenzo Bisceglie in Bari (Italy) encased mouse tumor cells in a nitro-cellulose membrane and inserted them into the abdominal cavity of a guinea pig, in which he showed that the cells were not killed by an immune reaction in the pig [1], various polymeric membranes have been used for the compartmentalization of many organs. These include islets for artificial pancreas, liver cells for bio-artificial liver, epidermal cells for artificial skin and osteoblasts for bone regeneration [2-5]. During the last few years neuronal cell behavior on biomaterial such as membrane has become of great interest since it offers the advantage of developing neuronal tissue that may be used for the *in vitro* simulation of human brain functions substitute. This could definitely provide further insights not only into the cell but also in developing therapies in neurodegenerative disorders such as Parkinson's or Alzheimer's diseases [6]. A bio-hybrid system using neurons could also represent a useful instrument for predictive drug testing or constitute a future model of a bio-neuronal network device. For typical neuronal tissue-engineered constructs, the properties of both cell (morphology, viability functions) and material (physico-chemical, morphological and transport properties) components are very important [7]. Current interest has been focused on attempts to find new biomaterials and new cell sources as well as novel designs of tissue-

engineered neuronal devices to generate safer and more efficacious restored neuronal tissue. Biomaterials that have been successfully employed in the manufacture of neuronal tissue include biodegradable materials such as polyglicolic acid, poly-L-lactic acid and poly (lactide-co-glycolide) and polymeric semi-permeable membranes (polyacrilonitrile-polyvinilchloride, polyethylenecovinylalcohol, cellulose acetate) in fiber and flat configurations [8-11]. Advances in polymeric chemistry have facilitated the engineering of synthetic membranes that can be specifically manipulated with regard to their physical and mechanical characteristics. The chemical nature and physical microstructure of substrates may influence the behavior of neurons.

Of all the brain areas playing an important part in numerous physiological activities, the hippocampus is recognized as an important telencephalic region, owing to its vital role in the consolidation of several forms of learning and memory and in particular in the formation of declarative memories. In this brain region, it is the pyramidal cell that has attracted a great deal of interest for neurobiological studies. These neurons have a well-defined shape and a distinctive dendritic arborization consisting of a single long apical dendrite and several shorter basilar dendrites, all greatly branched. In addition, pyramidal neurons are well-known for their plasticity and regeneration properties that have proved to be extremely useful in the unraveling of neuronal complex mechanisms following a stress condition or neuronal damages [12]. Neurodegenerative processes are becoming a major health concern especially since they are the cause of many neuronal disorders. In this context, the identification and establishment of molecular mechanisms involved in these processes have taken into consideration the evaluation of one of its specific markers, i.e. microtubule-associated protein 2 (MAP2), which has been demonstrated to be directly proportional to neuronal death [13]. Consequently, the application of a membrane bio-hybrid system as a potential biotechnological approach towards the regeneration of specific hippocampal neuronal networks may very well be suitable for screening cellular neurotoxicity and neuroprotection paradigms.

3.3. Membrane properties in a bio-hybrid system

When designing a membrane, special attention should be paid to the acquisition of well-defined morphology (pore size, porosity, roughness, thickness, etc.) together with desirable mass transfer properties. Various possible transport mechanisms and their combinations in real systems (from adsorption-diffusion transport in dense membranes to molecular sieving transport, Knudsen diffusion transport, selective surface adsorption, etc.) have been studied to describe the selectivity and permeability in dense and micro-porous polymeric membranes. In membrane bio-artificial organs using isolated cells as biological component, semi-permeable membranes carry out several functions: they act as immune-selective barriers for the transport of metabolites and nutrients from the medium to the cell compartment and of metabolites and specific products from the cell to the medium compartment. As a consequence, the membranes act as a physical and chemical support for cell adhesion, providing a large area for cell attachment, and also as a means of cell oxygenation [14, 15].

The transport of a chemical species across the membrane is a result of chemical potential differences (difference in temperature, pressure, concentration or combination of all these variables), which act on the various components of the system [16]. In accordance with the phenomenological approach of irreversible thermodynamics, the transport equations of solvent and solute can be expressed as the following:

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

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

Transport across a membrane is described by three parameters, hydraulic permeability Lp, solute permeability ω, and the reflection coefficient σ (eqs. 1 and 2), all being experimentally determined. The reflection coefficient is a measure of membrane selectivity and varies from 0 to 1 for a solute freely permeating and completely rejected by the membrane, respectively. The solute permeability coefficient is related to membrane solute diffusivity and thickness. In the absence of solutes, the equation becomes the following:

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

Lp is determined by measuring the solvent flux through the membrane under trans-membrane hydrostatic pressure differences. Lp is related to membrane properties such as pore size and the distribution of the different pore size, as well as pore geometry, thickness, and tortuosity. In porous membranes the transport of chemical compounds depends not only on the trans-membrane pressure gradient but also on the size and shape of the solutes related to the pore size in the membrane. The porous membranes used in bio-artificial organs are generally micro-filtration and ultra-filtration membranes: the pore sizes of micro-filtration membranes range from 10 to 0.05 μm, whereas ultra-filtration is typically used to retain macromolecules from a solution, since its lower limit consists of solutes with a molecular weight of a few thousand Dalton. When the hydrostatic pressure difference is zero the transport of a chemical specie through the membrane occurs across a concentration gradient according to the following formula:

$$J_s = \omega \Delta C \quad (4) \qquad \text{where } \omega = \frac{Deff}{l}$$

As a result, the transport of solutes is obtained as a difference in diffusion rates across the membrane arising from differences in molecular size. Overall, the size range and physico-chemical properties of solutes, which must be transported through a membrane in bio-artificial organs, are extremely variable. Small solutes such as electrolytes, oxygen and high MW proteins (70,000 Da) must be efficiently transported through the membrane as well as both hydrophilic molecules dissolved in the plasma and hydrophobic molecules. As a result, the transport of molecules across membranes depends not only on the size and physico-chemical properties of the molecules related to the pore size of the membranes, but also on solute-membrane interactions. In a tissue engineered neuronal construct, membranes can be used in a flat or hollow fiber configuration. In this case, hollow fiber membranes provide a wide area of cell adhesion in a small volume and a three-dimensional organization reminiscent of the tubular configuration of nerves (fig.1). Microporous polyethersulfone

membranes with 0.2 μm interconnecting pores have, in fact, been recently used as a scaffold to support the growth of human cells [17].

Flat membranes with a micro-porous structure are used not only for the adhesion of anchorage-dependent cells (e.g., neurons, endothelial cells, hepatocytes) but also for the transport of metabolites. On the other hand, dense membranes are useful to provide gas to the cells because of their permeability to oxygen, carbon dioxide and aqueous vapor (fig.2). In these systems, cells come into contact with the membrane surface and this greatly influences the response of cell behavior towards the type of membrane used. For this reason membranes should be chosen not only on the basis of their separation properties but also on the basis of physico-chemical and morphological surface properties. Cell-membrane interactions have to be closely investigated so that where it is possible membrane physico-chemical and morphological surface properties can be improved [18, 19].

It has been shown that the morphology of a cell tightly adhering to its substrate changes in relation to the properties of the substrate. As a consequence, the maintenance of cell morphology, in the same manner as that of *in vivo* conditions, is functionally vital to cells. In particular, the roughness and pore size of polymeric membranes seem to play an important role since they have been shown to influence the viability and metabolic rates of cells such as isolated hepatocytes [19]. The wettability of the membrane surface has proved to be another factor that affects the growth of neurons from PC-12 cells [20]. Although the basis for this difference is still poorly understood, the probable reasons for this effect may be, in part, due to the modification of the native substrate that anchors these cells, since the amounts and conformation of adhesion proteins contained in the culture medium vary. Currently, the contact angle is often used as a physical parameter indicative only of the wettability surface, but it allows more quantitative information concerning the energy parameters of the final surface material to be obtained [21]. The contact angle can be applied to characterize surface free energy parameters, the free energy of interfacial interactions in polymeric substrates. These measurements might be a predictive index of their cyto-compatibility and/or tissue biocompatibility. Therefore pre-treatment of surface material might enable the adaptation of its surface free energy to biological requirements.

Modifications of surface membrane with the addition of well-defined molecules that can mimic the external cell environment show great promise for the *in vitro* reconstruction of engineered tissues. Biomaterials are being designed to maintain specific cellular functions at the molecular level [22]. By immobilizing specific proteins, peptides, and other bio-molecules on a material it is possible to mimic the extra-cellular matrix (ECM) environment thus providing a multifunctional cell-adhesive surface [23, 24].

3.4. Membrane preparation features

The use of membranes in tissue engineering and medical fields seems to require materials with high biocompatibility and bio-stability properties [25]. Novel membranes were prepared from a polymeric blend of modified polyetheretherketone (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 techniques. PEEK-WC is a chemically stable polymer with excellent thermal and mechanical resistance [26]. Owing to the lack of crystallinity, PEEK-WC is soluble in various solvents. This property allows its application in preparing membranes with differing features by using the direct immersion-precipitation method referred to phase inversion. This is a cheap and flexible method that guarantees membranes with various structural properties following kinetic and thermodynamic parameter controls [27, 28]. The polymer solution, which is thermodynamically stable, is subject to demixing. The solution is loaded on a suitable support and immersed in a coagulation bath lacking solvents. The precipitation of a casting polymer solution is achieved by the exchange of solvent and non-solvent containing solutions. During the quenching process, the polymer solution separates into two phases: a polymer rich solid phase, which forms the membrane matrix, and a polymer poor liquid phase, which forms the liquid filled membrane pores. The final membrane structure, that is obtained results from a combination of mass transfer and phase separation. PEEK-WC-PU membranes were prepared from 9% (wt/wt) PEEK-WC polymer, 5% (wt/wt) PU and 2%Tetrahydrofurane (THF) (w/w) in Dimethylformamide (DMF). Solid medical grade Pellethane® 2363-80AE (Dow Chemical Company, Netherlands) was dissolved in DMF at

T=50°C. Thereafter, PEEK-WC polymer and THF were mixed to a PU solution. After casting, the polymeric films were coagulated in a bath constituted of 30% DMF and 70% ETOH. The membranes were washed extensively with water and dried at room temperature [29]. These membranes offer the advantage of combining the properties of both polymers (biocompatibility, thermal and mechanical resistance, elasticity) with those of membranes such as permeability, selectivity and well-defined geometry. The membrane displays a long-term stable condition in aqueous medium and so it can be easily sterilized by autoclave. In addition to exhibiting high chemical and thermal stabilities, this membrane has a homogeneous porous and wet surface, as confirmed by a water contact angle of about 77.71°, which was constant at all times. These morphological and physico-chemical properties are also important for the permeability of liquids and this becomes an important feature for the transport of nutrients and the removal of metabolites in a bio-hybrid system.

3.5. Hippocampus and plasticity: functional and molecular aspects

Another type of tissue that has been handled in the bio-hybrid system is the mammalian hippocampal formation located beneath the cortical hemisphere of the brain. This structure receives its name from its curved shape that reminded some neuroanatomists of a sea horse and others of a ram's horn named after Ammon's horn. Of all the telencephalic regions, the hippocampus is one of those regions, like the cerebellum and olfactory bulb, whose internal circuits are organized in a highly distinctive manner. In this context, stereotyped microcircuits and local circuits are used not only for processing sensory signals but also for information related to higher brain regions. The hippocampus may be divided into two major parts: the "hippocampal region" (composed of the dentate gyrus and Ammon's horn) and the "retro-hippocampal region" (composed of the subicular complex and the entorhinal area). This telencephalic region is best viewed as a series of adjacent cortical strips that consist of parasubiculum, presubiculum, postsubiculum and subiculum; fields CA_1 CA_2 and CA_3 of Ammon's horn and dentate gyrus (fig. 3). Moreover, Ammon's horn is usually divided into four layers: stratum lacunosum-moleculare, the cell-sparse stratum radiatum, the cell-dense

pyramidal layer and the cell-sparse stratum oriens. Taken together these four layers contain the greatest number of pyramidal cells with respect to other hippocampal areas [30].

It is worth noting that the hippocampus, through the complex interaction with other limbic regions such as the cortex and amygdala, is recognized for its highly remarkable synaptic plasticity capacity such as long-term potentiation (LTP) [31]. This type of plasticity involves both functional and morphological changes of synapses through the actin-dependent activation of pre-synaptic silent neurons; a feature which relies on L-type calcium channels and protein kinase A signaling pathways [32]. Such mechanisms prove to be essential for the explication of some key neurophysiological functions, such as memory and learning, as demonstrated by neurological damage to the hippocampus provoking memory deficits [33]. Although there is various evidence to underline the functional importance of hippocampal formation, specific contributions of this region as well as to the related structures such as the entorhinal cortex, the dentate gyrus, the individual CA fields and the subicular complex still remains a matter of dispute. As a consequence, some neuropsychologists propose their role in cognitive mapping and scene, declarative and relational memories [34] plus in the rapid acquisition of conjunctive associations [35]. In addition to this functional complexity, neuronal network modelling studies indicate that its intrinsic anatomy and synaptic physiology could mediate the rapid encoding and distributed storage of a large number of arbitrary associations.

Among the homogeneous population of hippocampal neurons, the pyramidal cells that are typical of CA_1 and CA_3 regions differ from other neurons not only for their physiological features but also for their well-defined shape and a distinct dendritic arborization consisting of a single long apical dendrite and several shorter basilar dendrites, all greatly branched. These functional and molecular characteristics make pyramidal cells extremely useful in providing further insights into complex neuronal mechanisms, such as plasticity and the susceptibility to anoxia, which are two major neuronal functions linked to glutammatergic and histaminergic activities [36, 37]. As matter of fact, recent works have demonstrated that histaminergic neurons protect the developing hippocampus from neuronal damage, with regulation of neuronal survival being mainly mediated by some specific histaminergic

receptor subtypes (H_nR) such as H_1R and H_3R [37]. In the case of the glutammatergic system, the numerous pyramidal synapses feature some AMPA (GluR1 and GluR2) and NMDA receptors (NR1 and NR2), which are activated during the induction of LTP and consequently permit an influx of calcium that triggers, in turn, the activation of this same neurosignaling pathway [38]. Interestingly, during the promotion of LTP, pyramidal spines show a notable increase in volume due to the accumulation of AMPA receptors and this might indicate an extraordinary remodelling property of pyramidal cells which is strictly related to plasticity-inducing stimuli [39]. On the basis of the above considerations and owing to the widespread connection existing between the hippocampal pyramidal neurons and other neuronal populations, such as endogeneous interneuron culture cells of such neurons that lack extrinsic afferent fibers, we are able to investigate not only the functional consequences of their reciprocal synaptic connections but also of their neurophysiological properties.

3.6. Neuronal cell isolation and culture on membranes

A prerequisite for the development of the neuronal membrane bio-hybrid system is the optimization of cell isolation and culture viability. A protocol of isolation and culture of hippocampal neurons has been optimized by using a hibernating rodent, the hamster *Mesocricetus auratus* as our animal model. Owing to neuroprotective adaptations such as hypothermia, metabolic suppression and increased antioxidant defense adopted during the various hibernating states, this rodent is a useful model to provide new insights into neurodegenerative disorders such as ischemia, which occurs during the arousal bout in hibernators [40].

The hippocampus of both hemispheres was isolated in a 1-2 days old hamster, removed and collected in falcon tubes in Neurobasal medium A (Gibco) containing 0.02% BSA. The hippocampal tissues were digested in a Neurobasal medium A containing papain 0.1% (Sigma) and BSA 0.02% (Sigma) for 20 min at temperature of 37°C. For this part, papain was used since it has proved to be an effective enzyme in dissociating neural tissue more than trypsin and at the same time it is less toxic to the neurons of the central nervous system [41]. Ten minutes after digestion, the tube containing the hippocampus was mixed and at the

end of digestion, the supernatant containing papain was removed and to the remaining pellet Neurobasal medium supplemented with B27 (2% v/v) (Gibco) penicillin-streptomycin (100 U/ml), glutamine (Biochrom AG) 0.5 mM, B-FGF 5ng/ml (Sigma) was added. The samples were gently dissociated by pipetting up and down with 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 Neurobasal medium A 1% trypsin inhibitor and 1% BSA. This step was repeated twice, thereafter the samples were centrifuged at 1800 rpm for 10 min at room temperature. Cell pellets were gently re-suspended in Neurobasal medium containing B27 supplement, penicillin-streptomycin, glutamine 0.5 mM, B-FGF 5ng/ml. Serum-free B27 supplemented neurobasal medium seems to have a beneficial effect on the growth and differentiation of hippocampal neurons as suggested by other researchers [42-44]. The viability of the cells after this isolation procedure assessed by trypan blue test was shown to be 97±2%. In order to inhibit the growth of glial cells, cytosine arabinofuranoside at a final concentration of 1 μM was added at day 2 of culture and removed at day 4. The substrate is of great importance for the survival and differentiation of neuronal cultures. As substrate we used poly-L-lysine–coated Petri dishes and PEEK-WC-PU flat polymeric membranes. Poly-L-lysine (MW 30000-70000) dissolved in a boric acid/sodium tetraborate solution (1:1) to a final concentration of 0.1 mg/ml was plated onto Petri dishes at 4 μg/cm^2 density. For this part the density of plating appears to be extremely important because it influences the capacity of neuron survival. As a matter of fact high cell densities could lead to the apoptotic cellular death whereas at low cell densities (1500-5000 cell/cm^2) neurons have less cell-cell interaction [45] and do not receive autocrin neurotrophic factors from neighboring neurons that are necessary for a longer survival period [41]. In our study we used seeding densities in a range from 16000 cell/cm^2 to 40000 cell/cm^2 in order to define the optimal cell density. At high cell density (40000 cell/cm^2), cells after 4 hours adhered at the surface forming clumps and showing lamellipodia (Fig. 4a) while after 4 days cells undergo apoptosis and only debris are observed to cover the surface (Fig.4b). This is probably due to a competition for space required by the cells during their orientation towards the correct polarity as well as during

the formation of their processes such as dendrites and axons. At a density of 16000 cell/cm^2, after cells have adhered to the substrate, at day 4 we observed a flattening of the cells and minor processes starting to emerge from several sites along the circumference of the cells (Fig. 5a). With the progress of their growth procedures, the tiny neuronal filaments begin to acquire the definite characteristics of dendrites and axons and subsequently the development of synaptic contacts in this rich neuronal network (Fig. 5b). The complexity of the neuronal network increased within time: dendrites emerging from the cell body became highly branched (Fig. 5c). These results demonstrated that the plating density of 256000 cell/cm^2 is optimal for the survival and differentiation, at least of this type of neurons.

3.7. Selective assay of neurodegeneration: microtubule-associated protein 2

In the culturing of nerve cells, the development of immunocytochemical markers that selectively stain axons or dendrites has been of particular help to identify the *in vitro* different maturational stages of neuronal processes. Of all the possible biomarkers, the microtubule-associated proteins (MAPs) seem to play important roles in the morphogenesis, function and maintenance of the central nervous system [46]. A first and perhaps very important role of these proteins is their ability to promote microtubule polymerization and stabilization by maintaining the cylindrical form of neuronal processes [47]. Such a regulation of cytoskeletal elements is crucial in neurogenesis and these results imply a direct role of MAPs in neuronal plasticity. From a specific functional point of view, MAP2 is the most abundant in the brain and several works have been reported that the activation of kinase- and site-specific phosphorylation of MAP2 may be important in the regulation of MAP2 binding to microtubules and other proteins and hence may provide a mechanism for rearranging the specific structural constituents of neurons. Moreover, since MAP2 activity is closely related to the NMDA neurosignaling system, the onset of NMDA-dependent CaMKII activity has also led to the phosphorylation of MAP2, which in turn plays a key role in regulatory functions of the neuronal cytoskeletal components [48]. During development, MAP2c is expressed early during embryogenesis and gradually disappears after postnatal day 10 as neurite outgrowth declines and the cytoskeleton becomes more stable. However, in

regions of the adult brain undergoing continuous neuronal remodeling, such as the olfactory system, MAP2c levels remain elevated. These studies indicate that MAP2c exerts a major role in the conservation of neuronal cytoskeleton plasticity plus its requirement for neurite extension as demonstrated by the *in vitro* suppression of neurite outgrowth by specific MAP2c anti-sense sequences [49]. In our membrane bio-hybrid system, following the establishment of the optimal plating density of hippocampal cells and the evaluation of some properties such as adhesion and development of dendrites and axons on such a system, the cytoskeletal features were considered by investigating the distribution pattern of MAP2. A number of works have reported that cytoskeletal damage represents a crucial event in the degradation process following ischemia. MAP2 has been postulated to be a very early marker of ischemia neuronal injury, since loss of MAP2 immunoreactivity has been demonstrated to be directly proportional to neuronal death [12, 50]. The immunostaining method of MAP2 handled on pyramidal cell populations was conducted by using primary and secondary antibody conjugated with TRICT fluorochrome on samples previously fixed and permeabilized [45, 51]. In particular, the bio-membrane system in which neuronal cells exhibited a good level of adhesion was fixed with a solution of paraformaldehyde (4%) plus saccarose (2%) for 20 min at 4°C. Fixed cells were permeabilized and blocked with PBS buffer containing Triton (0,1%) and BSA (10%) for 30 min at room temperature. Cultures were then rinsed three times with PBS and incubated with the monoclonal antibody anti-MAP2 (1:100) overnight at 4°C. The following day, cells were rinsed with PBS and incubated with TRITC conjugated antibody (1:100) in PBS for 60 min at room temperature. The culture cells were counterstained with DAPI (200 ng/ml) and visualized by confocal laser microscopy images. The results obtained on membrane samples show a localization of this protein in the soma and in the emerging process after 48 hours (Fig. 6a). After 128 hours all cells developed well-defined dendritic processes, which were characterized by a high and intense distribution of MAP2. At day 18 the MAP2 becoming restricted to the proximal part of dendrites as expected and no loss of MAP2 was detected in these cells (Fig. 6b). These results suggest that morphology and physiological properties of hippocampal pyramidal cells, which appear to be very sensitive to an inadequate culture media, have been not altered on

membranes and this relationship strongly supports the value of such a bio-hybrid substrate for long-term survival of neuronal cells and this means a more favorable condition to handle their neuro-transcriptional events within time.

3.8. Concluding remarks

This study reports on the potentiality of applying membrane bio-hybrid system in neural tissue engineering, evidencing the crucial points in the *in vitro* reconstruction of the physiological neuronal model. A number of issues need to be addressed: morphological and physico-chemical properties of the membrane, the optimal density of immobilized cells, the interaction of cells with the membrane and the viability and differentiation of neuronal cells in *in vitro* membrane constructs. Preliminary results demonstrated the feasibility of culturing hippocampal neurons in a membrane bio-hybrid system. Similarly to neuronal cell behavior on poly-L-lysine substrate, these cells adhere and organize to form a differentiated neuronal structure on membranes. The MAP2 immunoreactivity displays the unaltered cytoskeletal characteristics of cells after their differentiation and maturation process. The main finding of this study encourages the development of membrane engineering system of hippocampal neurons that are able to remodel and regenerate neural tissue in a well-controlled microenvironment. In a future perspective, this experimental system might be a valuable model to investigate complex neuronal networks existing between some major neurotrasmitter systems such as the histaminergic and glutammatergic system. Moreover, the identification of new cellular markers of neurodegeneration, such as specific factors involved in proteolytic activities may offer a new strategy for delaying axonal degeneration or neuronal loss of activity in neurodegenerative disorders [52].

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List of symbols

C = solute concentration, M L^{-3}

$\bar{c}_s$ = mean logarithmic concentration, M L^{-3}

D_{eff} = solute effective diffusion coefficient, L^2T^{-1}

l = membrane thickness, L

Lp = membrane hydraulic permeability, T L^{-1}

P = hydrodynamic pressure, M L^{-1} T^{-2}

Js = solute flux, M L^{-2} T^{-1}

J_V = volume flux, M L^{-2} T^{-1}

π = osmotic pressure, M L^{-1} T^{-2}

σ = Stavermann's coefficient, -

ω = solute permeability coefficient, LT^{-1}

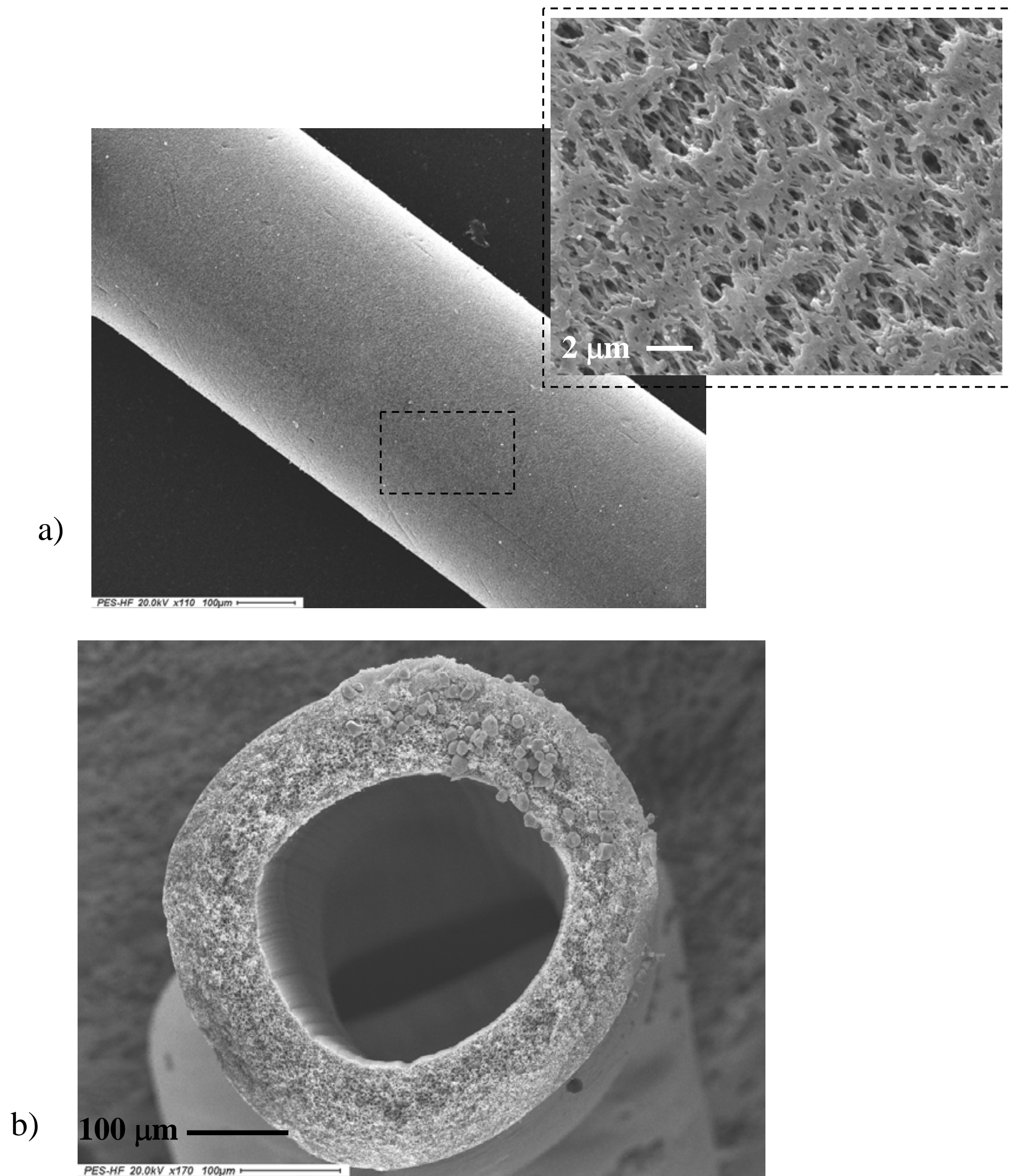


Figure 1. Scanning electron micrographs of polyethersulphone hollow fiber membrane: a) outside surface, b) cross section of the membrane.

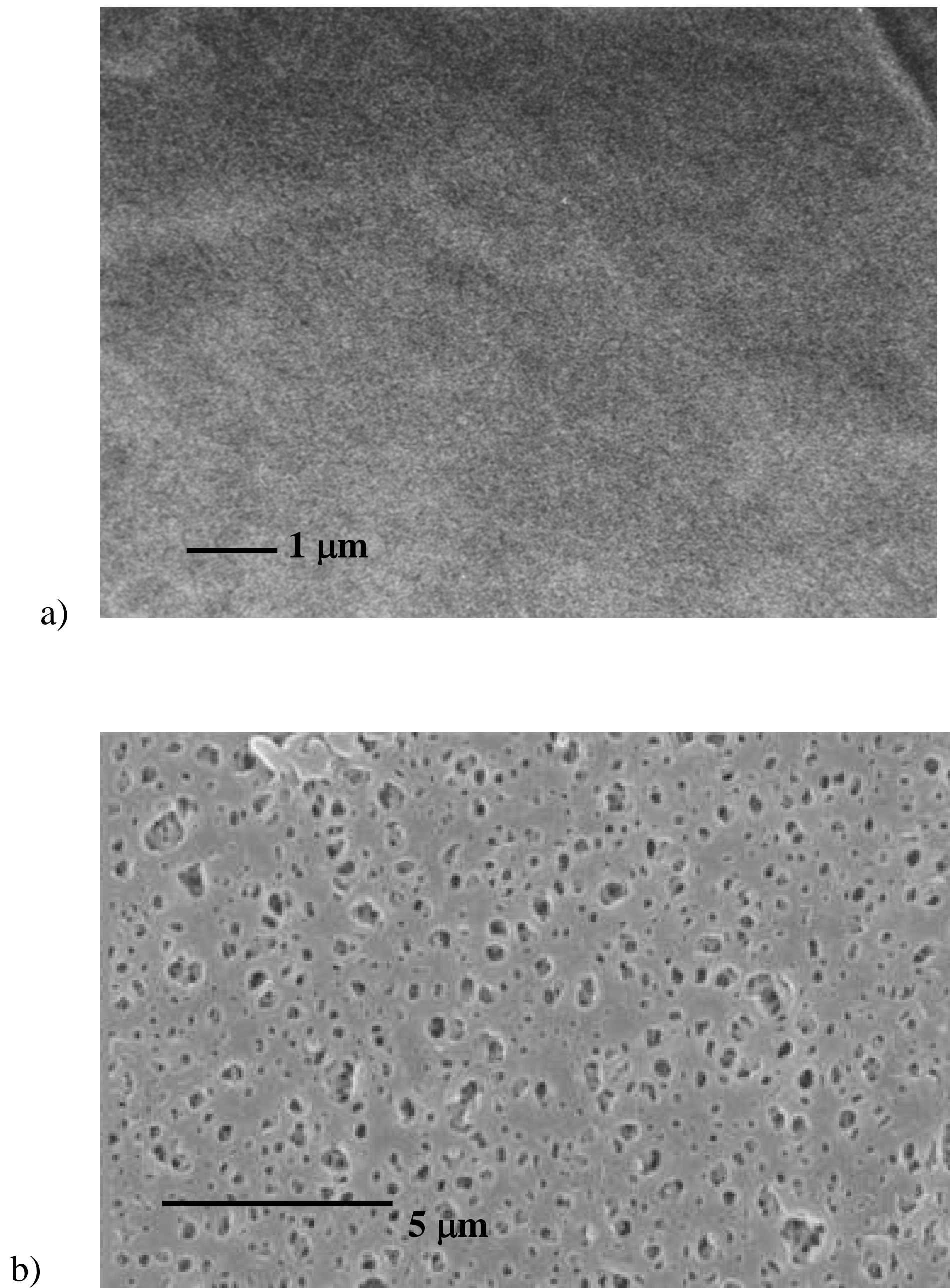


Figure 2 Scanning electron micrographs of flat membrane surfaces: a) polytethrafluoroethylene dense membrane; b) polyethersulphone microporous membrane

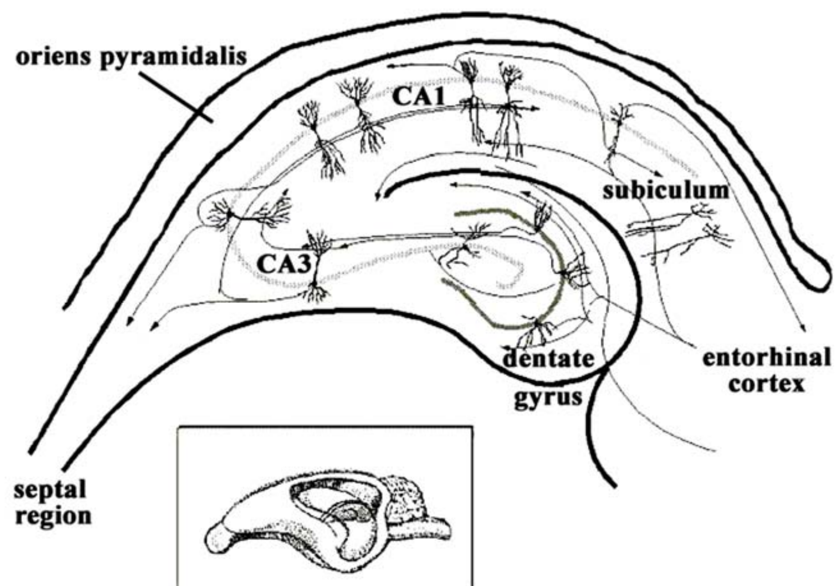


Figure 3. Anatomical structure of hippocampal formation in mammals.

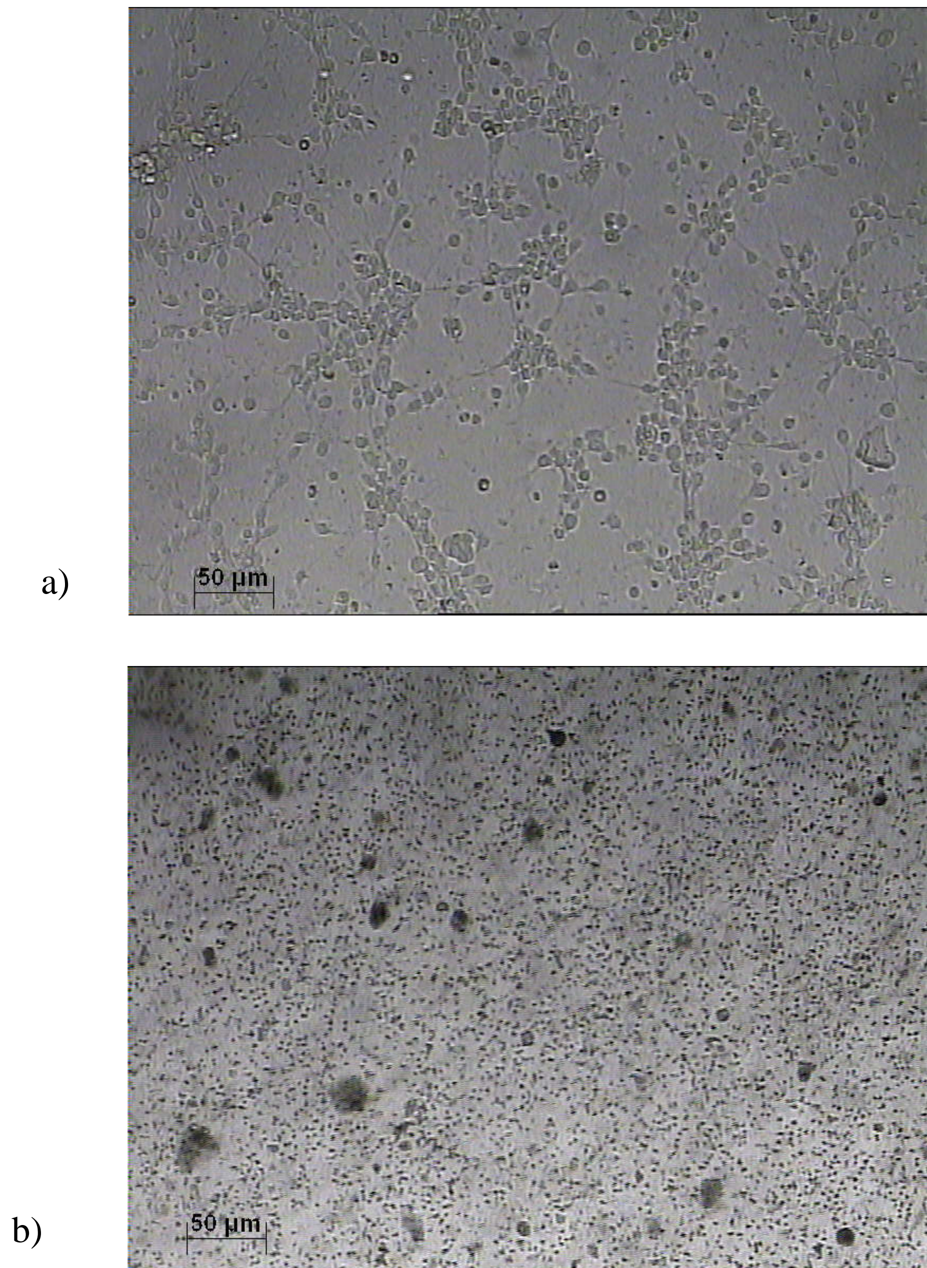
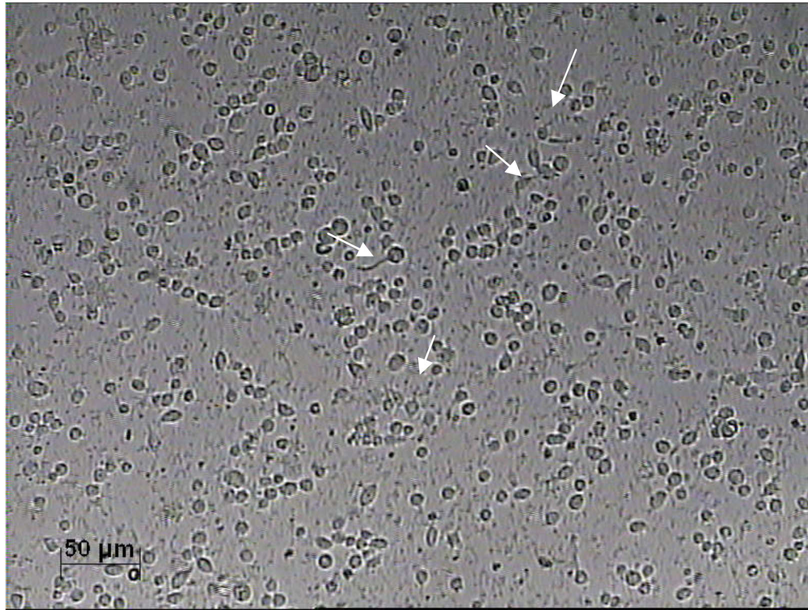
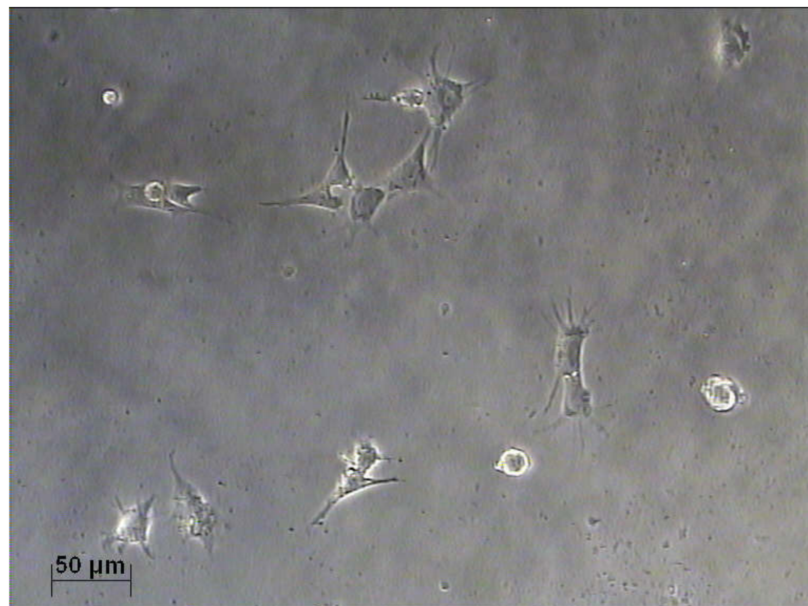


Figure 4. Micrographs of hippocampal neurons at seeding density of 40000 cell/cm^{2}at on poly-L-lysine coated Petri dishes after a) 2 hrs of culture and b) 2 days of culture.



a)



b)

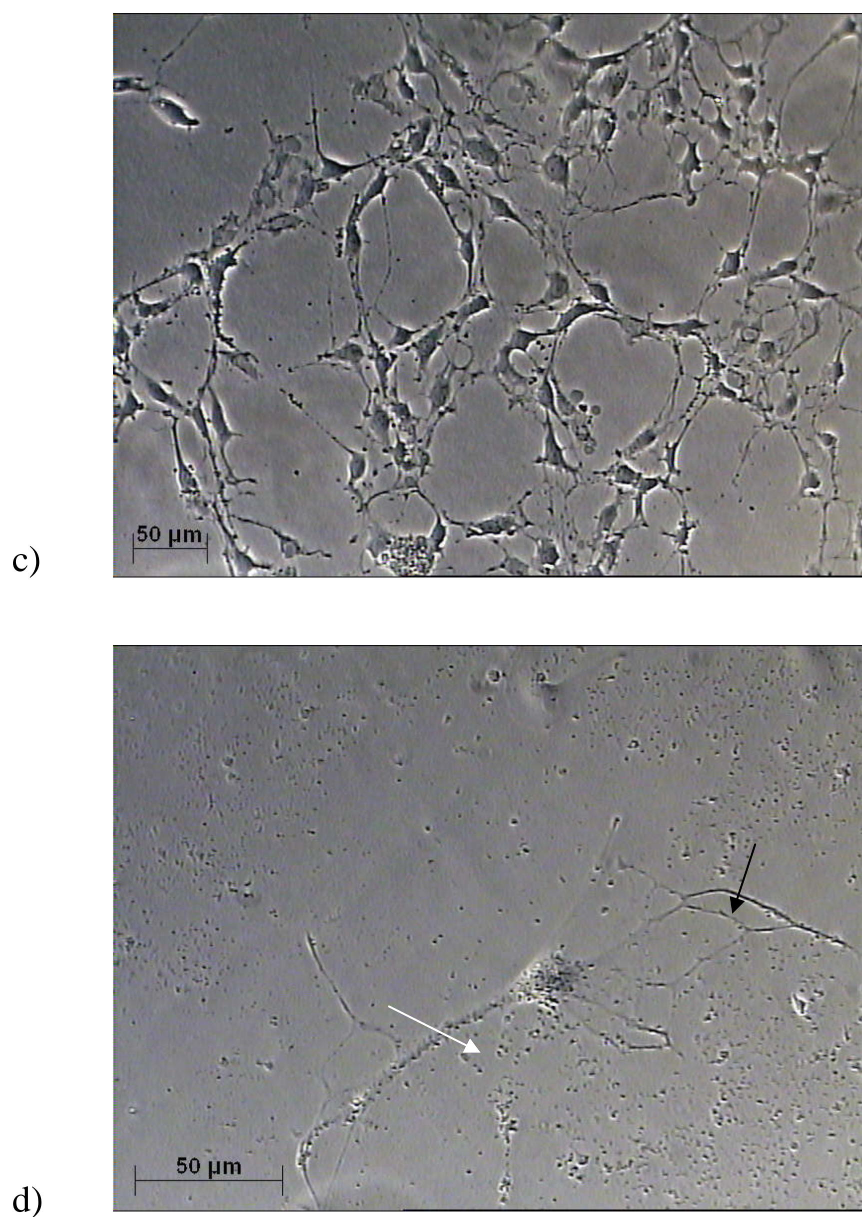
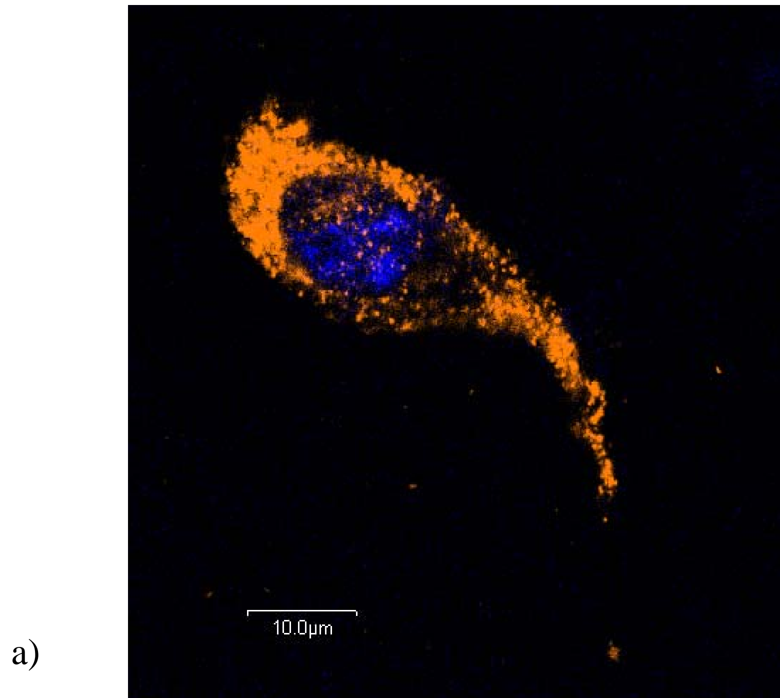
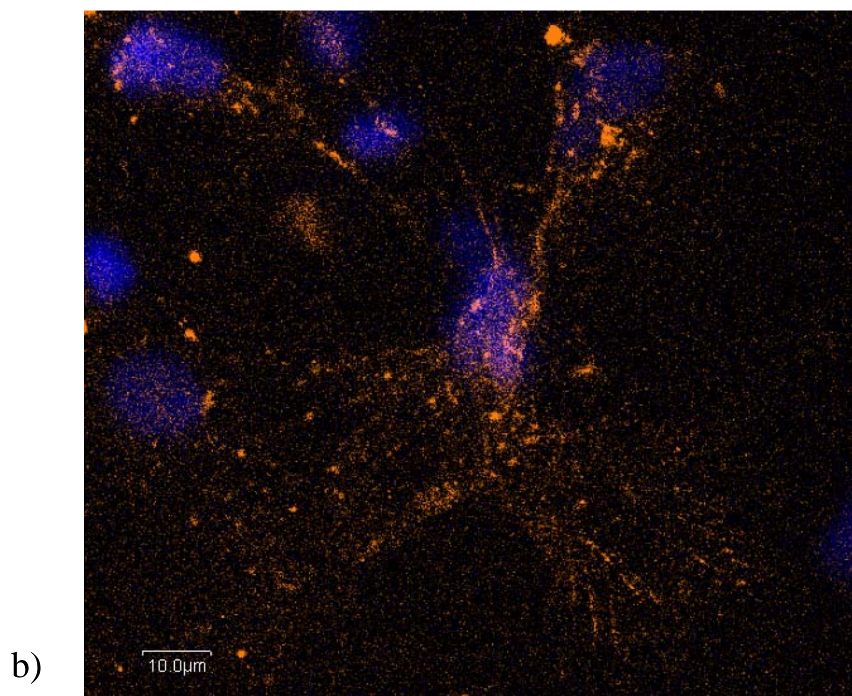


Figure 5. Micrographs of hippocampal neurons at seeding density of 16000 cell/cm^2 on poly-L-lysine coated Petri dishes after a) 4 hrs of culture; b) 2 days of culture; b) and c) 12 days of culture. The arrows in a) and b) indicate the emerging processes from the cell circumference; the arrows in c) indicate the (white) axon and the branched dendrites (black).

a)



b)



c)

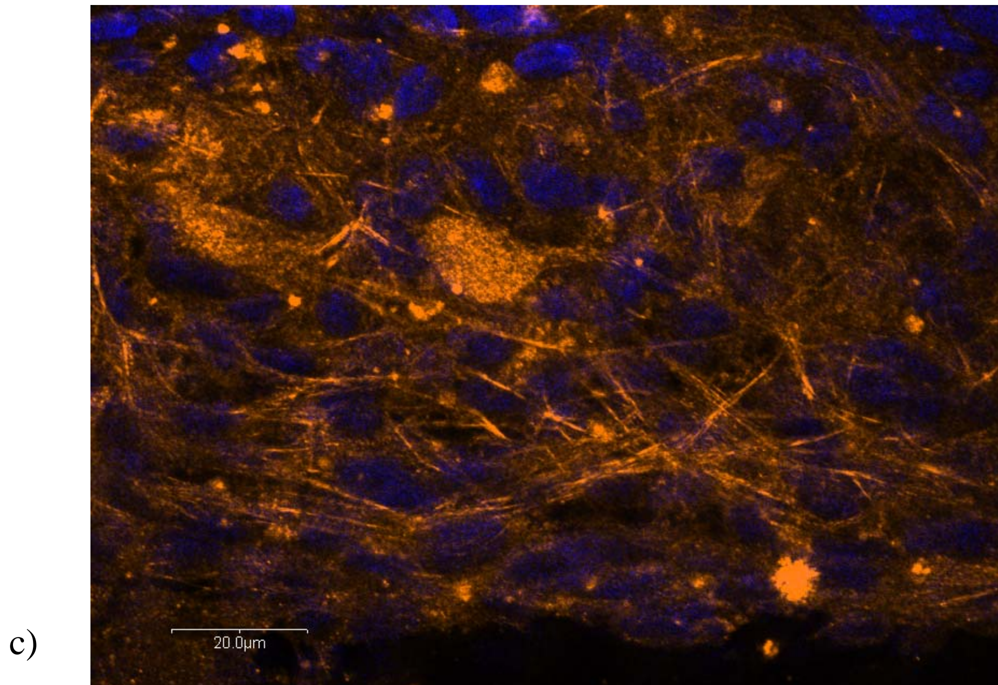


Figure 6. Confocal laser micrographs of hippocampal neurons a) after 4 days of culture on PEEK-WC-PU membranes; b) after 18 days of culture on PEEK-WC-PU membranes; c) after 18 days of culture on poly-L-lysine coated Petri dishes. The cells were immunolabeled for MAP 2 (orange) and cell nuclei were labeled with DAPI (blue).

Chapter 4

Chapter 4

Influence of membrane surface properties on the growth of neuronal hippocampal cells

4.1 Abstract

Membranes have become of great interest for tissue engineering application, since they offer the advantage of developing neuronal tissue that may be used in implantable or *in vitro* hybrid systems for the simulation of brain function. The behaviour of neurons isolated from the hippocampus on membranes with different surface properties was investigated.

The different membranes used as substrates for cell adhesion consisted of polyester (PE), modified polyetheretherketone (PEEK-WC), fluorocarbon (FC) and polyethersulfone (PES), all of which coated with poly-L-lysine (PLL) in order to have the same functional groups interacting with cells. The membranes exhibited different morphological surface properties in terms of pore size, porosity and roughness.

Hippocampal neurons exhibited a different morphology in response to varying the properties of the membrane surface. Indeed, cells grown on the smoother membranes and namely FC and PES membranes displayed a large number of neurites with consequent formation of bundles. 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. In addition, the secretion of brain-derived neurotrophic factor (BDNF) was expressed at high levels in neurons grown on FC membranes with respect to the other membranes. Taken together these results suggest the pivotal role played by membrane surface properties in the adhesion and growth of the hippocampal neurons, which must be considered in the development of tailored membranes for neural tissue engineering.

4.2 Introduction

Advances in neural tissue engineering require a comprehensive understanding of neuronal behaviour on biomaterials, which provide mechanical support and guide cell growth in a

new tissue or organ. Current interest is focused on attempts to find new biomaterials and new cell sources as well as novel designs of tissue-engineered neuronal devices to generate healthy and more efficacious recovered neuronal elements [1-2]. Among the biomaterials that have been successfully applied in the manufacture of neuronal tissue, polymeric semi-permeable membranes can be used as support for the adhesion of neurons and as a selective barrier for the transport of nutrients to the cells and for the removal of catabolism waste products in implantable or in vitro hybrid system [3]. Advances in polymer chemistry have facilitated the engineering of synthetic membranes that can be specifically manipulated with regard to their physical and mechanical characteristics, which may affect the interactions with cells.

In particular, surface properties of polymeric membranes such as surface free energy parameters, roughness and pore size seem to play an important role since they have been shown to influence the viability and metabolic rates of other cells such as isolated hepatocytes [4-5]. With respect to the neural engineering applications, the wettability of the membrane surface has proved to be a factor that affects the growth of neurons from PC-12 cells and meningeal cells [6-7], modulating the adsorption of adhesion proteins contained in the culture medium or secreted by cells that mediate the adhesion of cells to the substrate. 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 [8-10]. Topographic guidance of neuritis outgrowth has been explored in vitro with culture substrate containing etches, microchannels, nanotubes or microgrooves [11-15]. Although the topographical influence on the neuronal adhesion and orientation has been investigated by using designed artificial substrates, the effect of microporous membrane surface properties on the growth of primary hippocampal neurons has not still been studied.

In this paper we describe our efforts to investigate how the membrane surface properties influence the behaviour of neuronal cells. We used neurons isolated from hippocampus, which is an important brain area owing to its vital role in the consolidation of several forms

of learning and memory and especially during the formation of declarative memories [16-17]. Hippocampal neurons, which are well-known for their plasticity and regeneration properties [18], are the best-characterised model for investigating polarization that occurs spontaneously during the first days of culture [19-21].

Polymeric membranes consisted of polyester (PE), modified polyetheretherketone (PEEK-WC), fluorocarbon (FC) and polyethersulfone (PES), with different morphological properties (e.g., pore size, pore size distribution, porosity and roughness) were coated with poly-L-lysine (PLL), in order to have surfaces with the same functional groups but with different morphological properties. PLL was chosen for coating because is the substrate that allows the in vitro growth of neuronal cells [22]. The membranes were tested to compare neuronal growth and metabolic behaviour of cells aimed to establish the membrane properties capable of reconstructing the neural network *in vitro*.

In this context, the quantification of brain-derived neuronal factor (BDNF) secretion in these bio-hybrid systems constitutes a key functional marker for developmental hippocampal properties, since this neurotrophic factor with specific cytoskeletal functions appeared to be also responsible for pre-synaptic dendritic arborization of embryonic hippocampal neurons [23]. The properties of membrane surface could be important elements for the application of the membrane bio-hybrid system in neuronal tissue engineering for in vitro investigation of in vivo environment. The membrane biohybrid systems could be potential valuable tools aimed to study and understand cellular mechanisms of neurodegenerative processes and for drug testing.

4.3 Materials and Methods

4.31 Membranes

Among the available commercial membranes we have chosen those membranes that were tested previously for culture of other anchorage-dependent cells [5]. Commercial microporous membranes such as Polyester (PE) (Osmonics, USA) and Polyethersulfone (PES) (Pall, USA) were used together with dense membranes such as Fluorocarbon (FC)

(In Vitro Systems & Services, Germany) membranes permeable to oxygen, carbon dioxide and aqueous vapour.

Membranes were also prepared from modified polyetheretherketone (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) by inverse phase techniques using the direct immersion precipitation method [24] PEEK-WC is a chemically stable polymer with excellent thermal and mechanical resistance [25]. PEEK-WC membranes were prepared from 10%.(wt/wt) PEEK-WC polymer, 24% (wt/wt) PEG (w/w) in dimethylformamide (DMF). After casting, the polymeric films were coagulated in a water bath at T=40°C. The membranes were washed extensively with water and dried at room temperature [26].

The 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 $\mu g/cm^2$. 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. FITC labelled PLL (Sigma) was used for the visualization and quantification of the membrane coating. Imaging of the FITC labelled coated membranes were obtained by using an Olympus Fluoview FV300 Laser Confocal Scanning Microscope (LCSM) (Olympus Italia). Quantitative analysis was performed on different area of 3 samples of each investigated coated membrane using Fluoview 5.0 software (Olympus Corporation) by measuring the average intensity of fluorescence. A calibration curve of FITC labelled PLL was obtained by casting known quantities of the fluorescent protein on defined areas of polystyrene dishes calculating the surface concentrations and capturing confocal images of the dry samples. The thickness of the PLL coating resulted to be 1.2 ±0.09 μm, as measured with FITC labelled PLL by Z-direction scanning at the LCSM.

4.3.2 Membrane characterisation

The morphological properties of the membranes were characterised in terms of mean pore size, pore size distribution, thickness and roughness.

Dried membrane samples were cut and mounted with double-faced conductive adhesive tape and analyzed by scanning electron microscope (Quanta 200F ESEM, FEI, USA). From some selected representative images it was possible to observe the typical morphology of the membranes and namely, the shape and size of membrane pores as well as the pore size distribution.

The roughness of the membrane surfaces after PLL coating was evaluated by using Atomic Force Microscopy (AFM), Nanoscope III (Digital Instruments, VEECO Metrology Group). Tapping Mode^{TM} AFM operated by scanning a tip attached to the end of an oscillating cantilever across the sample surface. The cantilever was oscillated at or near its resonance frequency with amplitude ranging typically from 20 nm to 100 nm. Silicon probes were used. Surface roughness was estimated with respect to the mean absolute value difference, Ra, and the root mean squared difference, RMS, between the actual surface height and that of the line dividing the surface of the investigated profile into two equal areas. The reported roughness values are the average of twenty measurements on different membrane samples.

The wettability of the membrane, which is an important parameter for cell adhesion, was characterized by means of water dynamic contact angle (DCA) measurements. The contact angle of water droplets was measured at room temperature with a CAM 200 contact angle meter (KSV Instruments LTD, Helsinki, Finland). DCA measurements were performed under standardised conditions, which take into account various parameters (e.g., temperature, cleanliness of sample, drop volume). The instrument supported by video camera and software permits precise drop measurements and evolution in time. DCA measurements were performed on native and PLL coated membranes. At least 30 measurements on different regions of each membrane sample were averaged for each DCA value. Standard deviations are indicated as error bars.

The permeability properties of the membranes were characterised by pure water flux measurements in the absence of solutes and at different transmembrane pressure (ΔP^{TM}). For each membrane the hydraulic permeance L_p, was evaluated before and after modification process with PLL by the following equation [27]:

$$L_P = \left(\frac{J_{Solvent}}{\Delta P^{TM}} \right)_{\Delta c = 0}$$

This equation assumes a linear correlation between water flux and the convective driving force.

4.3.3 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 [28]. 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 [29]. 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 [30, 22]. 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.

4.3.4 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 4 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 anti- 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 anti- 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.

4.3.5 Neuronal morphology features of the different 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 ± std.dev.).

4.3.6 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.*

4.3.7 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 ($p < 0.05$).

4.4 Results

The membranes displayed a different surface morphology as Fig. 1 shows. All membrane parameters are reported in Table 1. FC membrane exhibits a dense structure without any pore (Fig.1a). This membrane is permeable to oxygen, carbon dioxide and aqueous vapour. PES membrane surface shows pores with mean size of 0.1 μm (Fig. 1b) and with homogeneous pore distribution. The membrane has a porosity of 74.4±1.6% and a thickness of 140±0.5 μm (Table 1). Pores with round shape and mean diameter of 0.1 μm are regularly distributed over the PE membrane surface (Fig. 1c). These membranes are very thin as evidenced by the thickness value (Table 1). On the other hand PEEK-WC membranes have pores with an elongated shape and mean size of 0.2 μm (Fig.1d). The porosity of this membrane is also similar to that of the PES membrane. Membrane surfaces seem to maintain their morphological features after coating with poly-L-lysine as we can see from the SEM's images (Fig.1e-h).

The effective coating of the membranes with PLL was evaluated in the absence of cells by a quantitative analysis using FITC labelled PLL for detection purposes only. As observed in Fig. 2, the amount of PLL on the different membranes determined by measurements of the average fluorescence intensity was in the range of 40 $\mu g/cm^2$ and not statistically different among them confirming the similar coating level of all membranes with PLL.

The PLL coating of the membranes modified their native physico-chemical properties as expected (Fig.3). The advancing and receding contact angles for native FC membranes

and PEEK-WC decreased after PLL coating to values respectively of θ_{adv} = 64.3±4.1° and θ_{rec} 36.9±6.4 for FC and θ_{adv} = 71.3±6.1° and θ_{rec} 40.2±7.5 for PEEK-WC. Native PES membranes have a very high hydrophilic surface character in fact the water contact angle measured on this membrane was 30.6± 1.2°. Also the modified membranes display a marked wettability even if, in this case, the PLL coating induced a reduction in the surface hydrophilic character. The wettability of the PE membranes did not change significantly after PLL coating as show the values of water contact angles.

The topographical images of the modified membranes show surfaces with nanostructures, which confer rougher profile to the surfaces (Fig. 4). The FC (Fig. 4a) and PEEK-WC membranes (Fig.4d) appeared to be respectively smoother and rougher with respect to the other membranes. The evaluation of the average roughness reported in Table 1 confirmed the rougher profiles of the AFM images: in fact membranes have different values of average roughness ranging from 6 to 200 nm.

The membranes exhibited also different permeability properties, which are important for the transport through the membranes (Table 1). The observed steady-state hydraulic permeance of the investigated membranes was calculated accordingly as the slope of the flux J versus the transmembrane pressure (ΔP^{TM}) straight line. PE membrane was more permeable with respect to PES and PEEK-WC membranes. This result is in a good agreement with the small value of membrane thickness that for porous membranes is inversely related to the water flux. The coating reduced the hydraulic permeance of 64% for PE membranes and 20% for PEEK-WC membranes. Not significant changes were observed in the hydraulic permeance of the PES membranes after coating.

After characterization of morphological and permeability properties, the membranes were used for cell culture experiments. It is particular interesting to note that the isolated hippocampal neurons adhered successfully to the different surfaces. In Figs 5 and 6 are reported the micrographs of hippocampal neurons on FC membranes and on PSCD (control). Both substrates have characteristics of transparency therefore permitted the online observation of the cells with time by an inverse light microscope. During the first hours of culture a flattening of the cells was observed as well as minor processes starting to

emerge from several sites along the circumference of the cells (Figs. 5a and 6a). With the progress of their growth period (Figs. 5b and 6b), the tiny neuronal filaments begin to acquire the definite characteristics of dendrites and axons and subsequently the formation of synaptic contacts develop into a rich neuronal network (Figs. 5c and 6c). The complexity of the neuronal network increased with time: dendrites emerging from the cell body became highly branched (Figs. 5d and 6d).

Figure 7 shows the adhesion of the isolated hippocampal neurons to the different surfaces. On day 4 of culture the area covered by cells was moderately ($p<0.05$) higher for FC, PES membranes as well as PSCD, whereas neurons grown on PE and PEEK-WC substrates adhered to a lesser extent than that of the other membranes. Neurons on the FC membranes (Fig.8b) as well as on PSCD (control) (Fig.8a) developed axons and highly branched dendrites and formed a complex network of neurite bundles. A similar morphology was also displayed by cells on PES membranes: the cells formed an axonal network at high density and highly branched processes plus the development of large neurite bundles (Fig.8c). On the PE membrane surface, cells adhered forming processes over the membrane surface that established intercellular contacts (Fig.8d). Conversely, on the PEEK-WC membranes cells developed short neurites with the tendency to grow into the pores of the membrane surface (Fig.8e). The area covered by cells increased with time and after 16 days the FC and PES membranes similarly to PSCD supported the growth of hippocampal neurons (Fig.7).

The confocal images of the neurons on the different surfaces showed the localization of βIII-tubulin (green) in the neuronal network (Fig.9). 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. On FC (Figs. 9c-d) and PES membranes (Figs.9e-f) cells tended to show a somatic morphology that was comparable to that of neurons grown on PSCD substrates in spite of longer axons developed at 4 days of culture (Fig. 9a and 9e). Quantitative analysis confirmed that hippocampal axons were moderately longer on FC and PES membranes along with PSCD than those developed on the other two substrates after 4 days

(Fig. 10). Interestingly axonal growth continued in time above all for smooth surfaces as demonstrated by moderately longer axons with respect to rougher substrates after 16 days of culture (Fig.10). A complex neuronal network was achieved after 16 days of culture, as demonstrated by moderately longer axons measured on smoother membranes with respect to rougher substrates (Figs.9d and 9f). In the case of PE (Ra= 87.2nm), neuronal formations only developed above the membrane surface and the cells exhibited a good neurite outgrowth but a lower degree of fasciculation with respect to FC and PES membranes (Figs.9g-h). Conversely, in the case of PEEK-WC membranes (Ra=200 nm), the smaller cells with respect to other membranes tended to aggregate and develop their processes into the pores of the membrane surface (Figs.9i-l).

It is worthy to note that the altered levels of some of the major metabolic activities and namely glucose consumption and BDNF secretion support the conservation of vital cell function at different growth periods (Figs. 11a-b). A comparison of the glucose consumption of cells among the membranes showed high level of glucose consumption on day 4 of *in vitro* culture on FC, PES and PE membranes as well as on PSCD. Cells on PEEK-WC displayed low rate of glucose consumption. The glucose uptake decreased with time for all substrates reaching on day 16 values of 256 μg/mL millioncell and 242 μg/mL millioncell on FC and PES membranes respectively (Fig. 11a).The evaluation of the metabolic activity showed that cells maintained their function in terms of BDNF secretion at different levels throughout the entire culture period, depending on the membrane surface (Fig. 11b). The production of cellular BDNF was detected up to 16 days of culture. On FC membranes as well as on PSCD, cells exhibited significantly high levels of BDNF on day 4 and 8 of in vitro culture. The synthesis of BDNF on PSCD decreased from 51.8 pg/mL millioncell (day 4) to 3.9 pg/mL millioncell (day 16) whereas on FC the secretion of BDNF on day 16 was 17.55 pg/mL millioncell on day 16 of culture (Fig.11b).

4.5 Discussion

The main objective of this study was to compare the adhesion and growth of primary neurons isolated from the hippocampus on membranes with different surface properties. The membranes were modified by coating with PLL in order to minimize their native different physico-chemical properties and to have the same functional groups interacting with cells. Membranes after coating displayed different morphology in terms of pore size, porosity and roughness.

The dynamic contact angles of the native membranes evidenced the different physico-chemical properties of these substrates. As the advancing and receding contact angles are respectively a measure of the apolar and polar aspect of the surfaces, it is interesting to note the difference of these properties among the investigated membranes. The PLL coating led significant changes in the wettabilities of the membranes yielding more homogenous surfaces with respect to their native character. An appreciable hysteresis was also observed on the membrane surface, which is caused by the surface roughness, microporosity, the heterogeneous distribution of polar and apolar domains over the surface as well as reorientation of groups in the surface under the influence of the liquid phase.

The results of the membrane characterisation displayed the effective PLL coating performed on all investigated surfaces that shown a similar PLL density (Fig.2).

Neurons shown the capacity to respond to the different membranes, they adhered, polarized and developed neurites differently on the basis of the topographical characteristics of the surface. Neurons are unpolarised cells after isolation they polarized and establish axons and dendrites during the first days of culture (Figs. 5-6). Neuronal cells on the different membranes used in the present study displayed the typical morphology of hippocampal neurons that included a primary apical dendrite with multiple ramifications, very thin axons plus a discretely flattened soma. On membranes with surface roughness of 6 nm to 50 nm such as FC (Ra= 6.26 nm) and PES membranes (Ra=49.38 nm), neurons reached a well-developed state as shown by their large soma and ramification of their extending processes (Figs.8b-c and 9c-f). The density of axonal network increases the neuritis

become more elaborate and highly branched on the smooth surfaces. In the case of PE and PEEK-WC membranes, the neurons were less developed as demonstrated by the round shaped soma and poorly branched processes. Therefore the smooth membranes seem to be more supportive of neurite outgrowth modulating the development process of the neurons. Probably the roughness of the surface may either influence cell motility or hinder the extension and ramification of neuronal processes that emerge from the cell soma or guide the adsorption of adhesion proteins necessary for the interaction with membrane surfaces. Other studies in vitro support the importance of topography in the nerve growth and regeneration [31-32]. Neurons have been shown to adhere, migrate and orient their axons to navigate surface features such as grooves in substrates in the micro- and nanoscales [33]. Aebischer et al., [34] 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. 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 [35]. Our results obtained with hippocampal cells show an improved neuronal growth on smooth surfaces such as FC and PES membranes.

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, underlies the strong capability of FC and PES membranes to assure well defined plus, above all, orderly structured neuronal networks for a greater period of time more than that supplied by other substrates [36]. This constant expression of βIII-tubulin turns out to be very important since like others have reported cytoskeletal proteins are involved in basic cellular activities that include cell–cell interaction, cell adhesion and migration and any eventual damage may lead to the total degradation of neuronal processes [37-38]. It is worthy to note that even the axonal marker GAP-43, noted for the stabilization of actin in both long axons as well as the growth cone [39], resulted to be very intensely and

specifically distributed in the axon along with its growth cone, which further confirms its highly application value.

The evaluation of the metabolic activity of neurons on membranes demonstrated that cells adhered on the membranes are functionally active for 16 days of culture. Differences in cell metabolism in terms of glucose consumption and BDNF secretion were measured among the investigated membranes. Notable levels of glucose consumption considered throughout the entire culture period in this study, demonstrate that the cells adhering to the membranes are also functionally active. A low ability to uptake glucose was measured on PEEK-WC membrane on days 4 and 8 of in vitro culture. Indeed, both at the beginning of neuronal growth (day 4) and at the later phases (day 16), cells on FC, PES and PE membranes exhibited high glucose consumption. In this context the secretion of BDNF, mammalian neurotrophin belonging to a family of structurally related dimeric proteins, is a further confirmation of the viable status of hippocampal neurons throughout the culture period. Hippocampal neurons exhibited high levels of BDNF secretion on FC membranes where cells formed highly branched neurites and a more complex network. The secretion of BDNF was high up to 8 days of culture, which corresponds to the maturation stage of hippocampal cells. This was confirmed by the morphology of the cells that it is widely known that this class of mammalian neurotrophins expressed at the central nervous system level plays a critical role in the survival, differentiation and maintenance of specific neuronal populations [40]. As a consequence, the elevated production of such a cellular protein at the brain level accounts for its involvement in processes such as the enhancement of synaptic transmission and the expression of other neurotrophins [41]. Moreover this neurotrophin is distributed not only along dendrites and therefore located postsynaptically but also presynaptically along the axon.

4.6 Conclusions

This study reports the effect of the membrane surface properties on the development of neurons isolated from hippocampus and on the potentiality of applying membrane bio-

hybrid system in neural tissue engineering. Neuronal cells respond to the different membrane surface by changing their morphology and neurite outgrowth. Smooth membranes tend to strongly favor the formation of well-polarized neuronal structures as confirmed by βIII-tubulin immunoreactivity, which specifically points to the unaltered cytoskeletal features of cells throughout their development processes. In agreement with morphologically integral neurons, the specific metabolic functions as displayed by the elevated secretion of BDNF corroborate the high expression of neurotrophin secretion on a smoother membrane surface up to maturation stage of hippocampal cells (8 days). The main finding of this study encourages the development of a membrane engineering system of hippocampal neurons, which is able to remodel and regenerate neural tissue in a well-controlled microenvironment. From a future perspective view, the surface characteristics of the membrane to be used in a neural tissue engineered construct have to be precisely engineered to combine physical and chemical stimuli aimed to develop neuronal network.

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List of symbols

AFM = Atomic Force microscopy

BDNF = Brain-derived neuronal factor

b-FGF = Basic fibroblast Growth Factor

BSA = Bovine Serum Albumin

C = solute concentration, M L^{-3}

DAPI = 4'-6-Diamidino-2-phenylindole

DCA = Dynamic Contact Angle

DMF =Dimethylformammide

ELISA = Enzyme-Linked ImmunoSorbent Assay

FC = Fluorocarbon

FITC =Fluorescein isothyocianate

GAP43 = Growth-associated protein-43

J_V = volume flux, M L^{-2} T^{-1}

Lp = membrane hydraulic permeability, T L^{-1}

LSCM = Confocal Scanning microscope

P = hydrodynamic pressure, M L^{-1} T^{-2}

PE = Polyester

PEG = Polyethylene Glicole

PES = Polyethersulfone

PEEK-WC = Polyetheretherketone

PLL = Poly-L-lysine

PND = post natal days

PSCD = Polystyrene culture dishes

Ra =

SEM = Scanning Electron Microscope

Greek symbol

θ = Angle, °

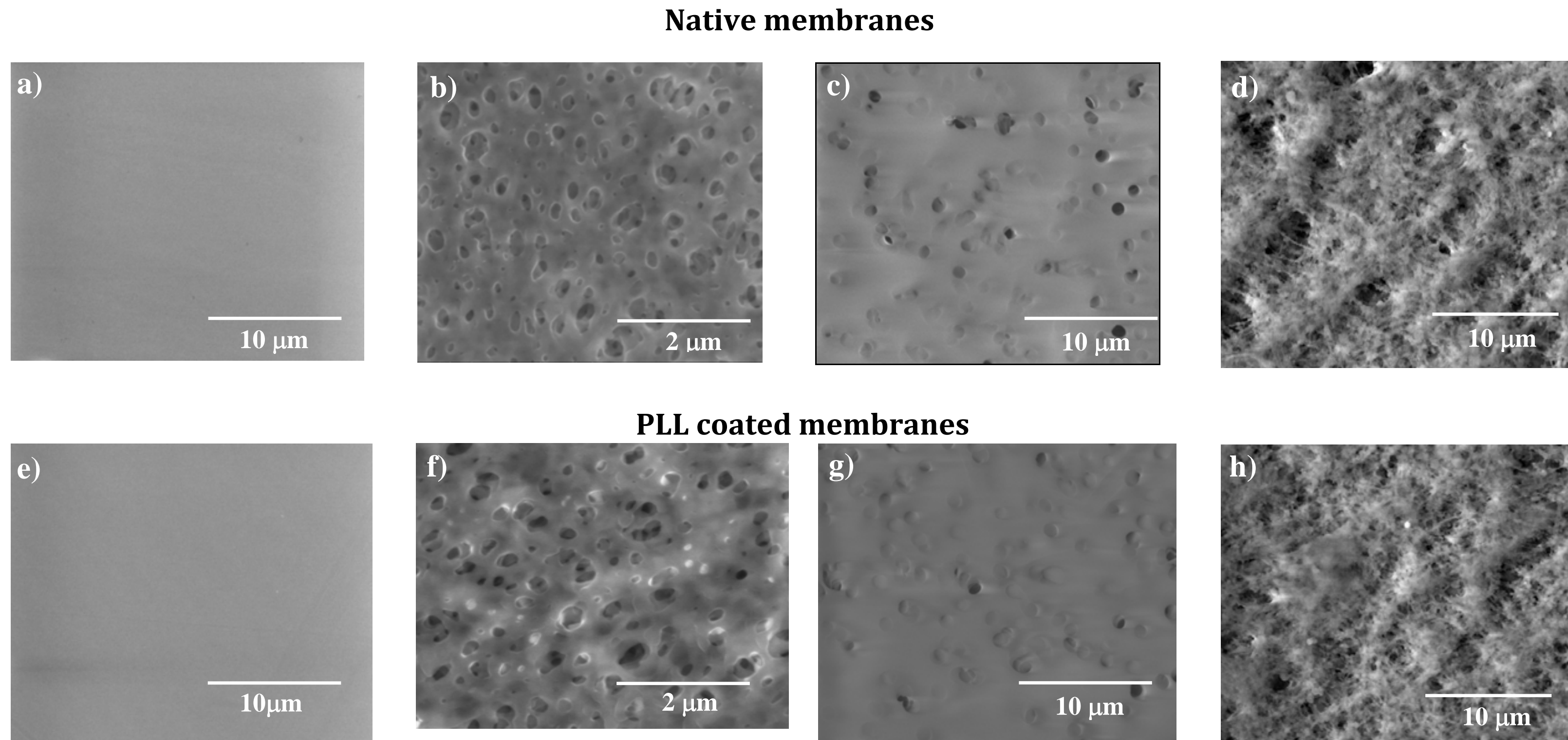


Figure 1 Scanning electron micrographs of native (a,b,c,d) and modified (e,f,g,h) membrane surfaces: a-e) FC, b-f) PES, c-g) PE, d-h) PEEK-WC.

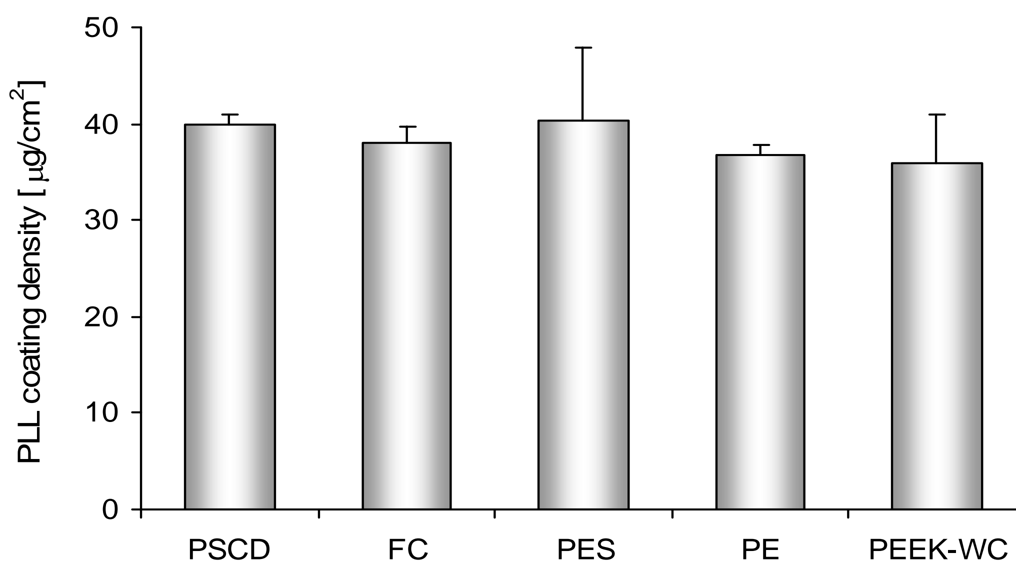


Figure 2 Poly-L-lysine (PLL) density measured on the different substrates after coating with FITC labeled PLL. The values were expressed as $\mu g/cm^2 \pm$ std.dev. and determined with respect to the fluorescence average intensity measured on PLL coated PSCD. Data were expressed as $\mu g/cm^2 \pm$ std.dev.

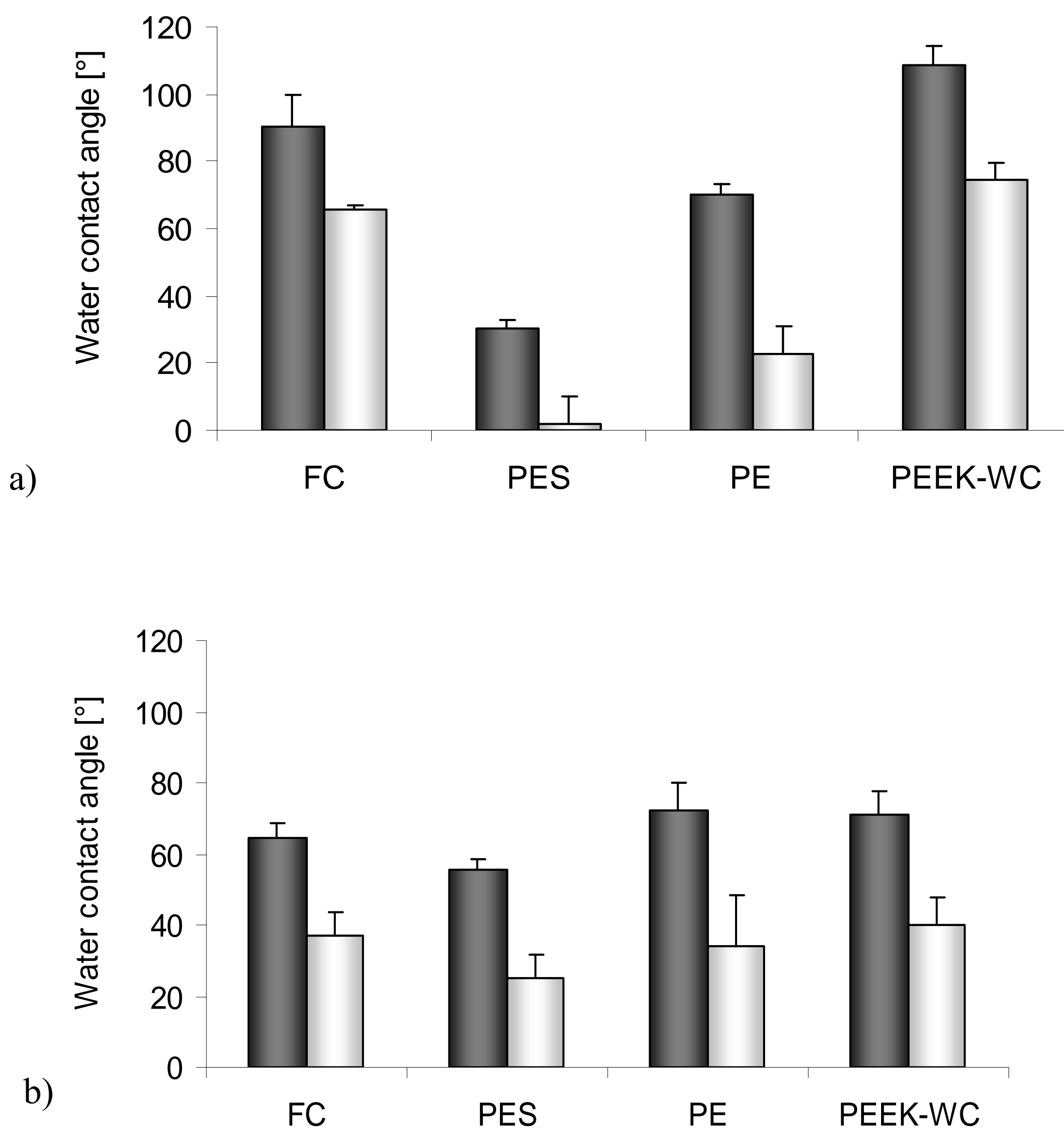


Figure 3 Advancing (full bar) and receding (empty bar) contact angle measured on: a) native membranes and b) PLL coated membranes.

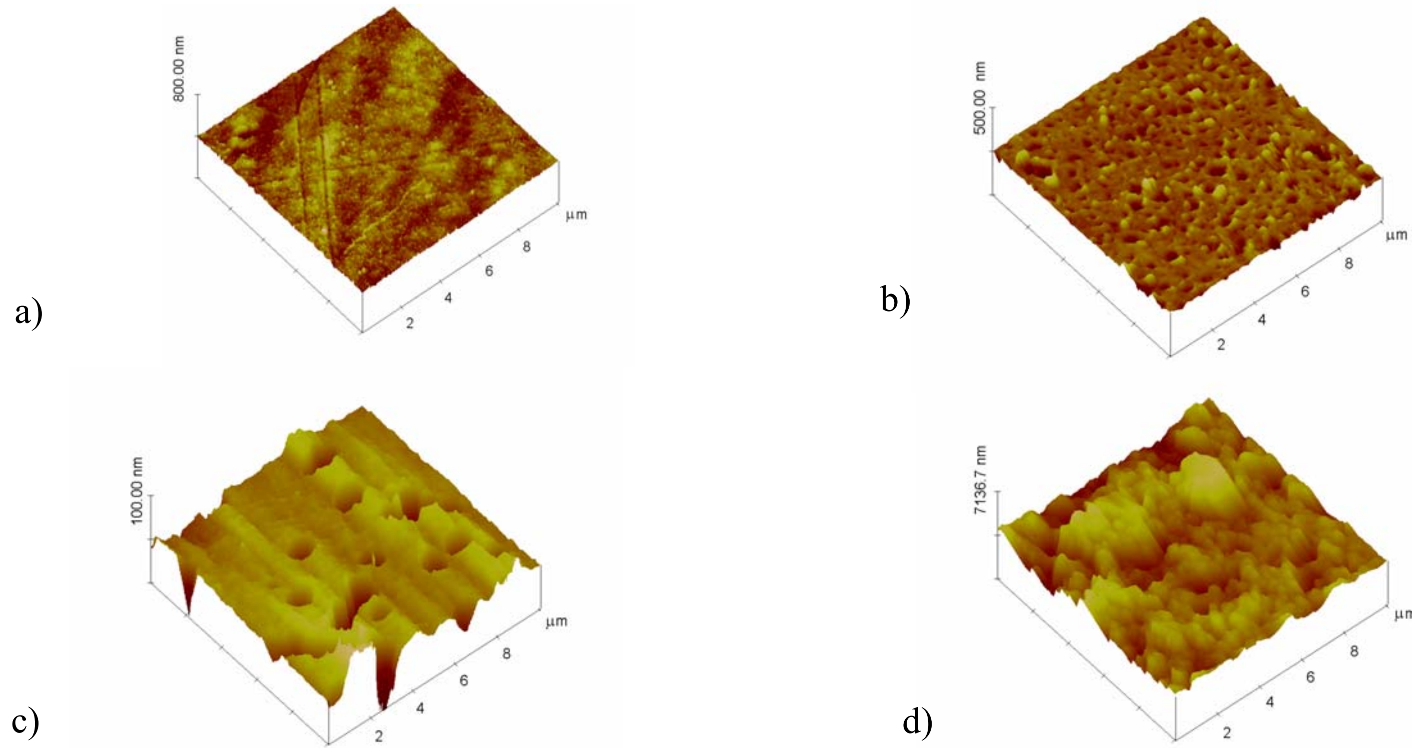


Figure 4 AFM micrographs of the modified membrane surfaces: a) FC, b) PES c) PE d) PEEK-WC.

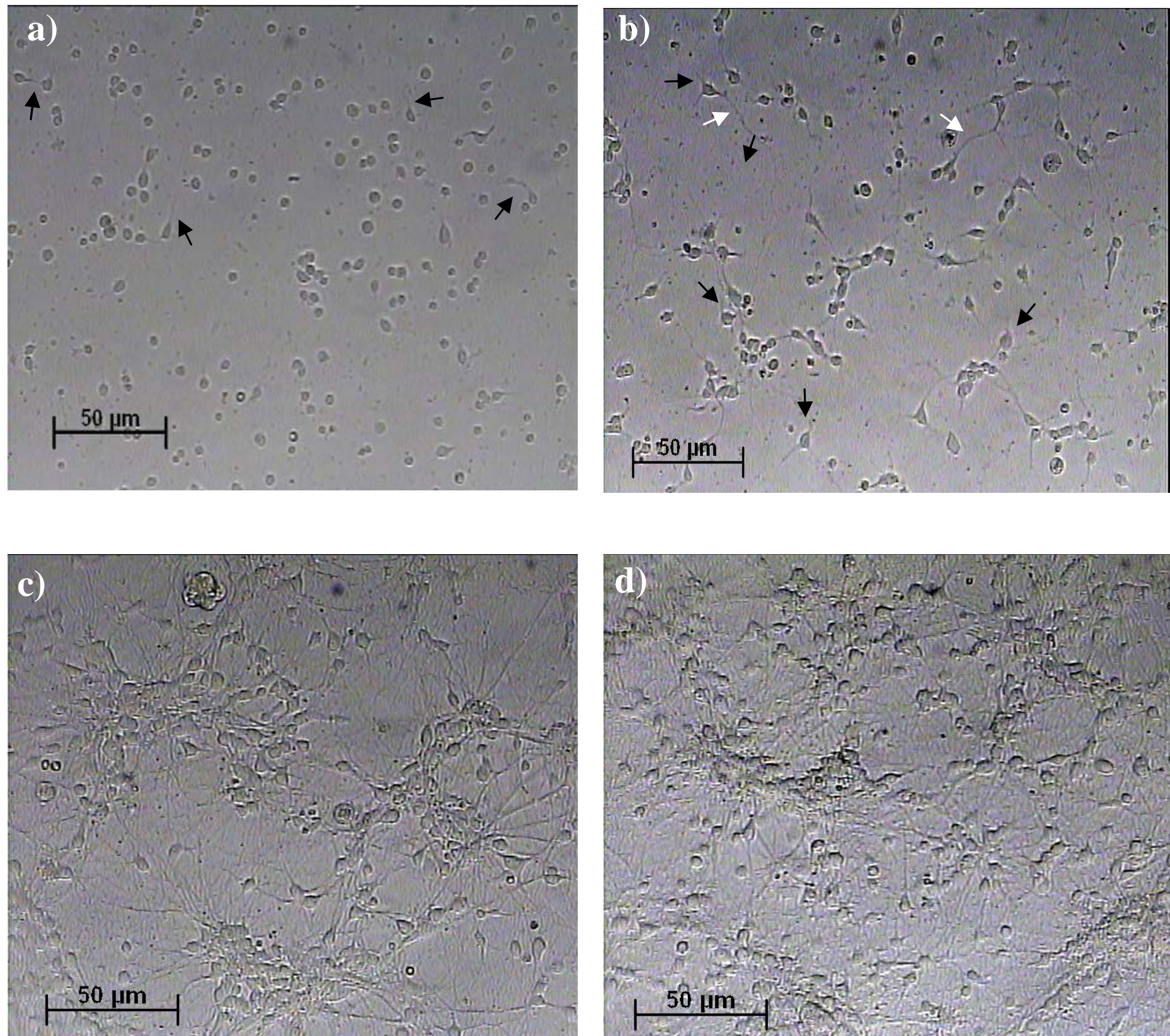


Figure 5 Micrographs of hippocampal neurons on FC membrane after a) 4 hrs, b) 3 days c) 8 days, d) 16 days of culture. The arrows in a) indicate the emerging processes from the cell circumference; the arrows in b) indicate the axon (black) and the dendrites (white).

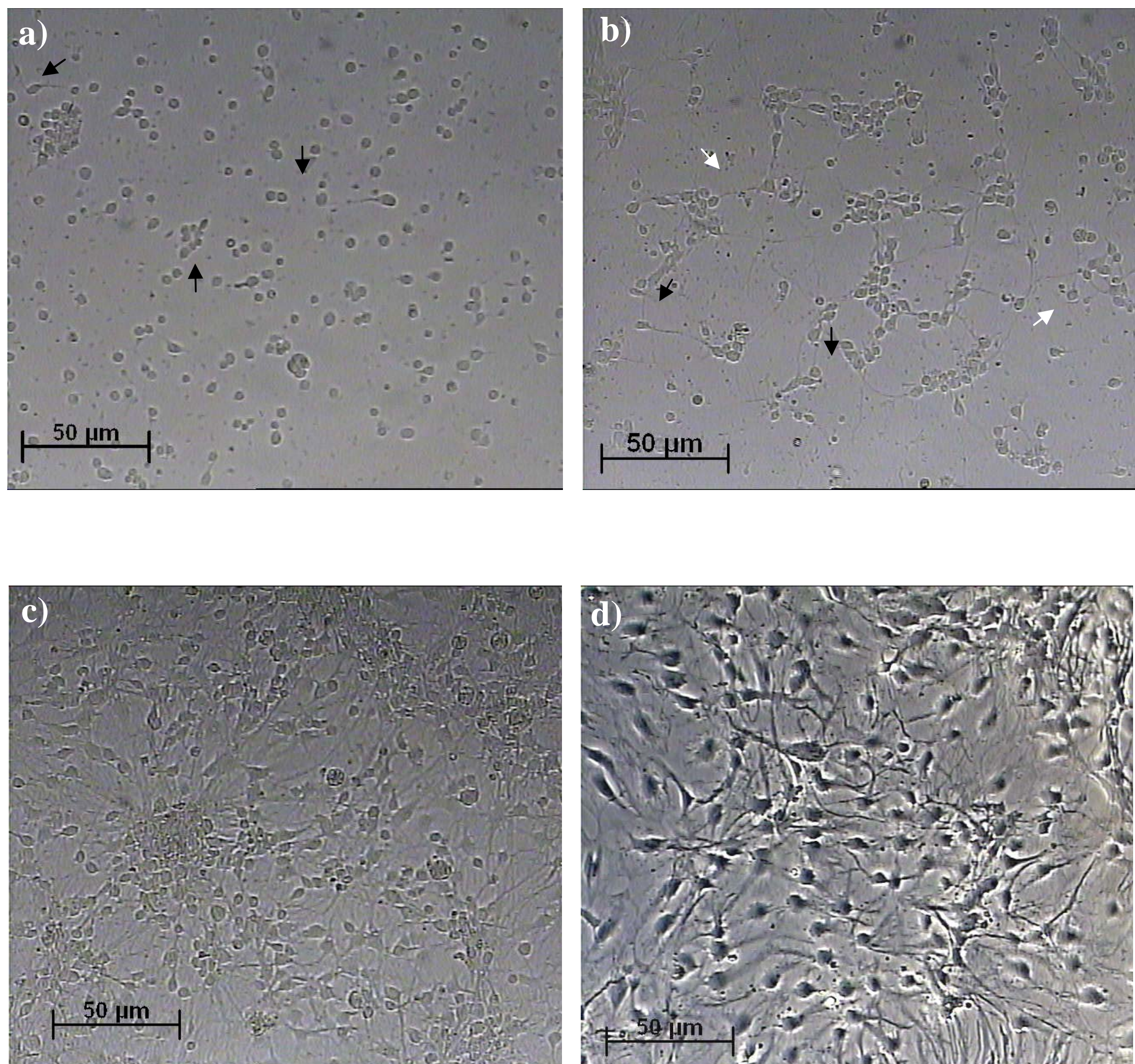


Figure 6 Micrographs of hippocampal neurons on PSCD after a) 4 hrs, b) 3 days c) 8 days, d) 16 days of culture. The arrows in a) indicate the emerging processes from the cell circumference; the arrows in b) indicate the axon (black) and the dendrites (white).

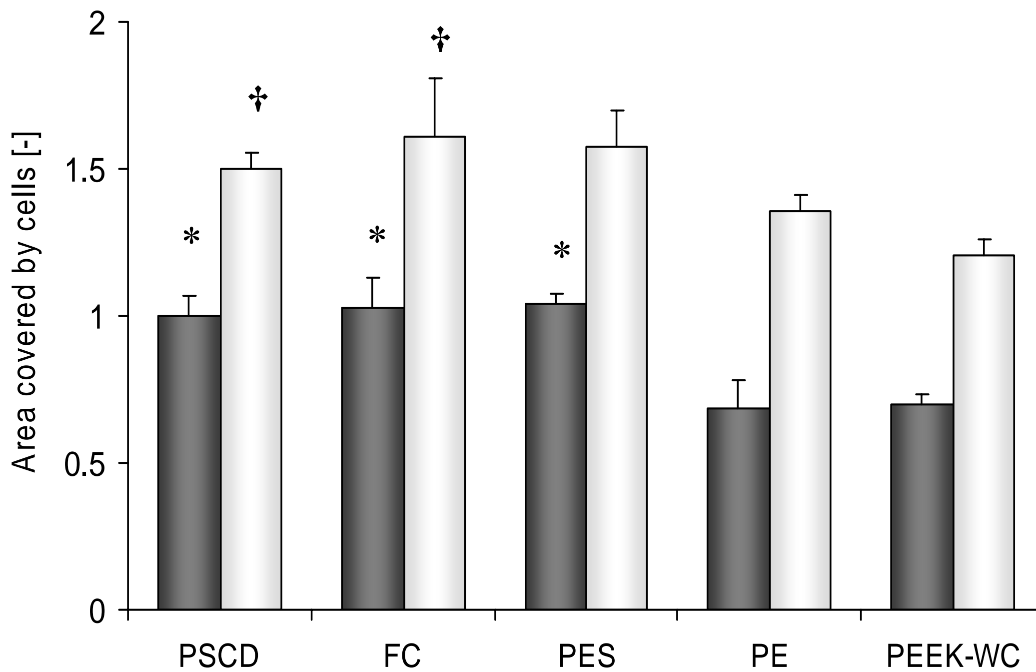


Figure 7 Area covered by hippocampal neurons after 4 (full bar) and 16 days (empty bar) of culture on the different surfaces. The data normalised with respect to the value of PSCD were expressed as average ± std.dev. and evaluated according to ANOVA followed by Bonferroni *t*-test. *$p<0.05$ versus PE and PEEK-WC membranes; †$p<0.05$ versus PEEK-WC membranes.

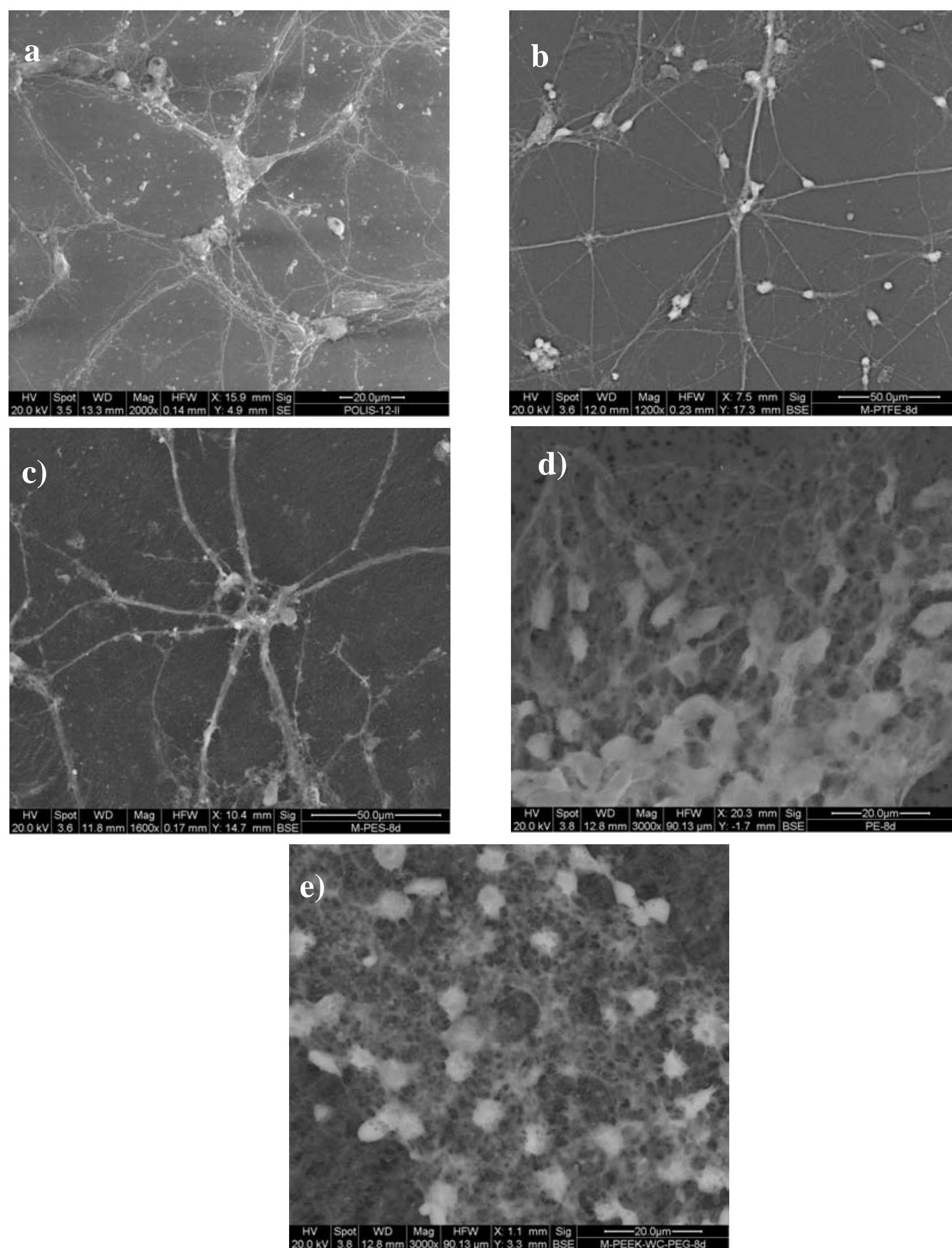
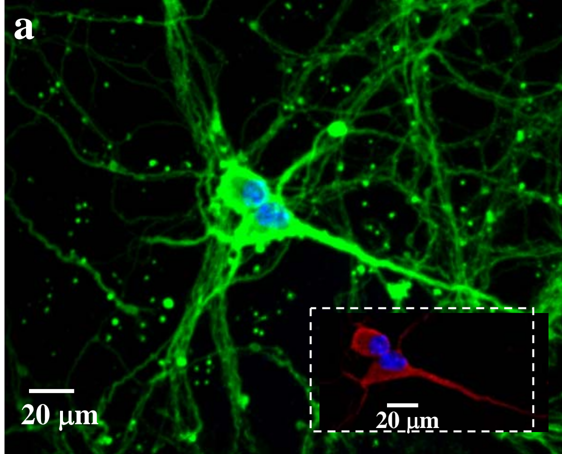
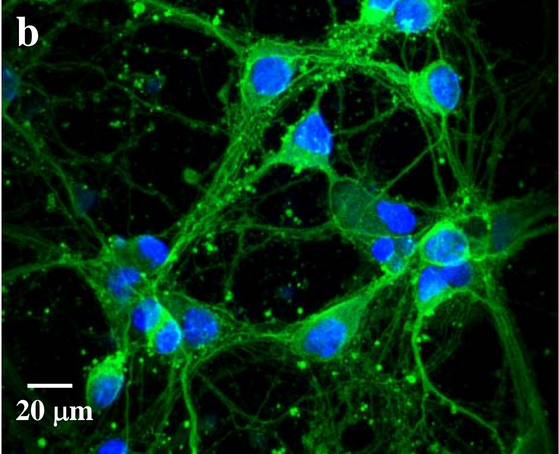


Figure 8 SEM's images of hippocampal neurons on: a) PSCD, b) FC, c) PES, d) PE, and e) PEEK-WC after 8 days of culture**.**


a
20 µm
20 µm


b
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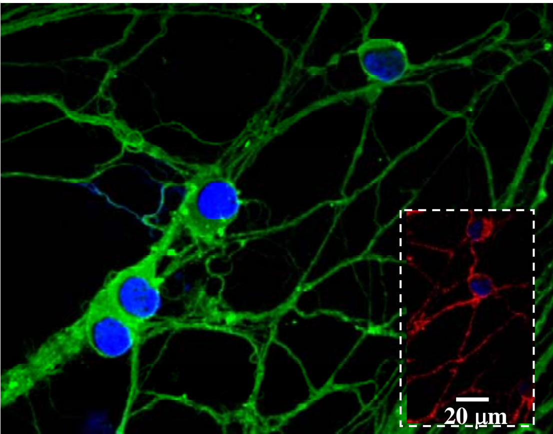
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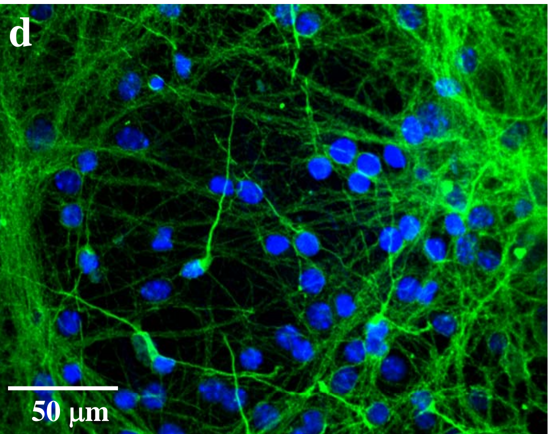
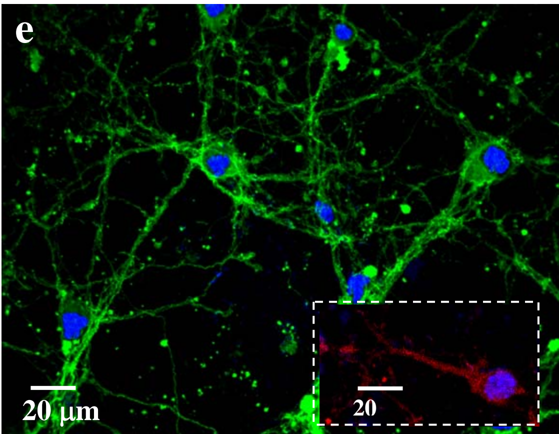

d
50 µm


e
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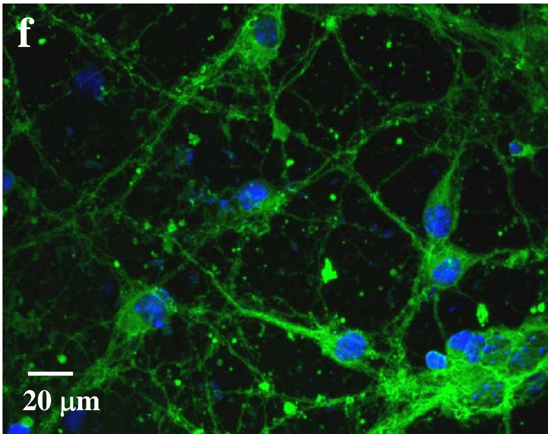
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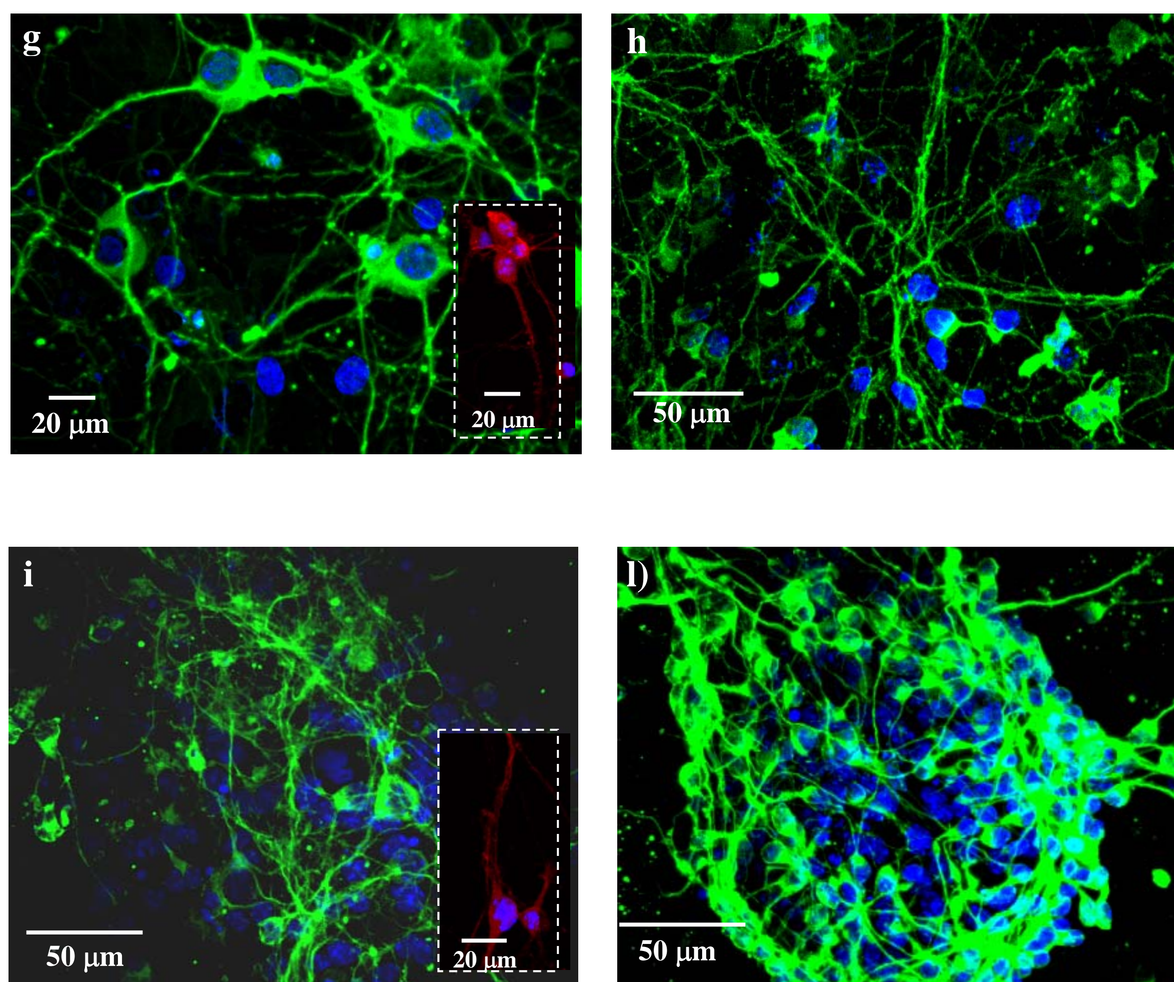


Figure 9 Confocal laser micrographs of hippocampal neurons traced for entire culture period and from representative images after 4 days (a, c, e, g, i) and 16 days (b, d, f, h, l) of culture it was possible to evaluate the distribution of βIII-tubulin (green) and the axonal marker GAP-43 (red) on: a-b) PSCD, c-d) FC, e-f) PES g-h) PE and i-l) PEEK-WC membranes. Cell nuclei were labeled with DAPI (blue).

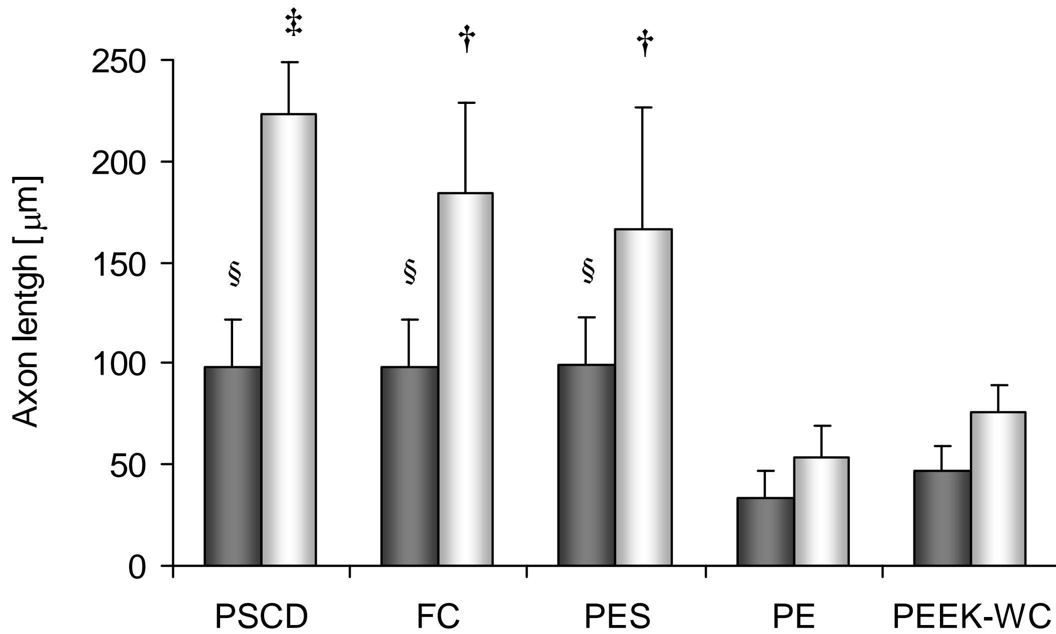
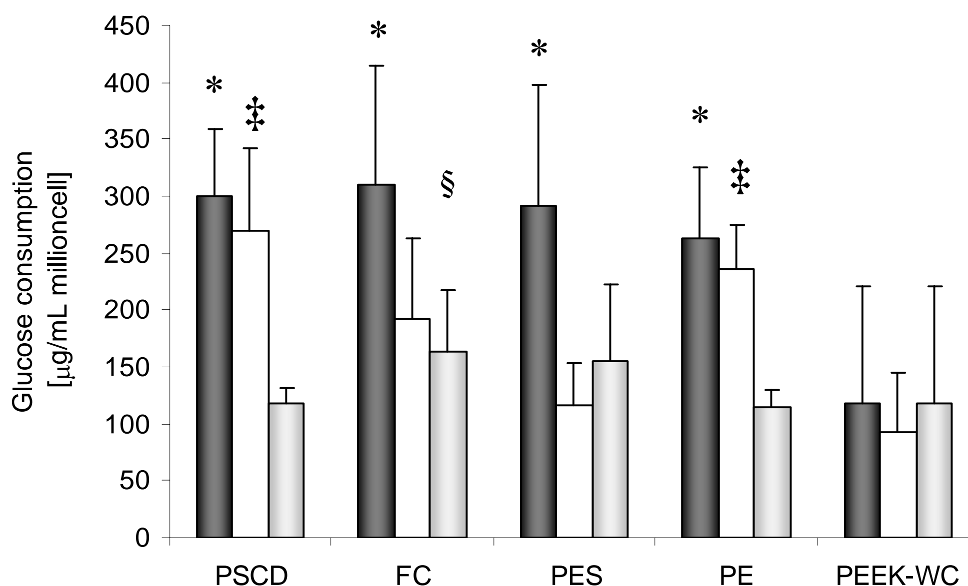


Figure 10 Axonal length of hippocampal neurons after 4 (full bar) and 16 days (empty bar) of culture on the different surfaces. Data were expressed as μm ± std.dev. and evaluated according to ANOVA followed by Bonferroni *t*-test. $^{\S}p<0.05$ versus PE and PEEK-WC membranes; $^{\dagger}p<0.05$ versus PE and PEEK-WC membranes; $^{\ddagger}p<0.05$ versus all.



a)

Figure 11 Metabolic activity of hippocampal neurons cultured on the different membranes.

a) 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 ANOVA followed by Bonferroni *t*-test. *p<0.05 versus PEEK-WC; ‡p<0.05 versus PES and PEEK-WC; §p<0.05 versus PEEK-WC.

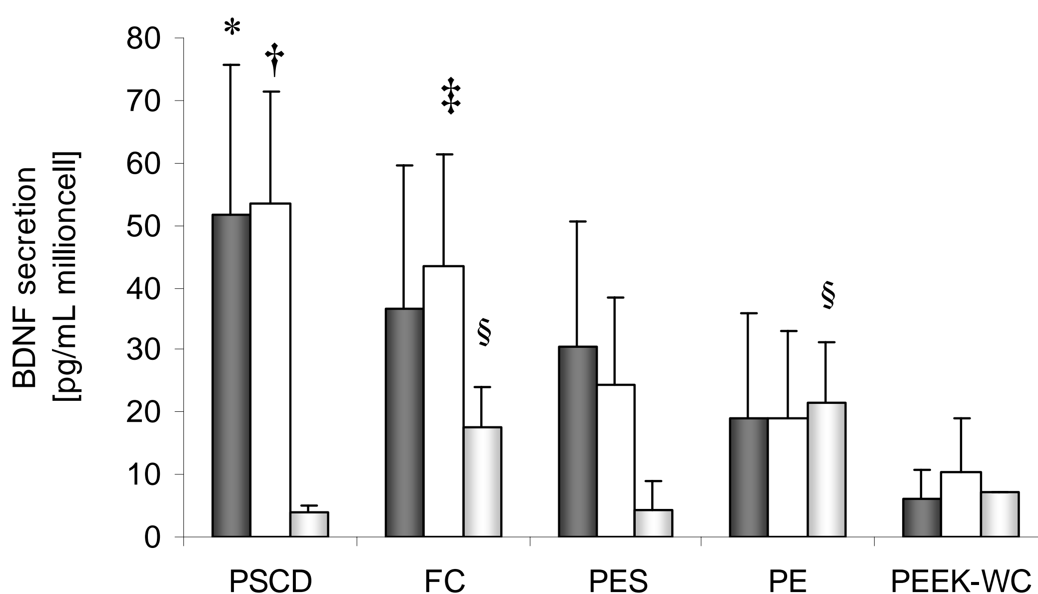


Figure 11 Metabolic activity of hippocampal neurons cultured on the different membranes.

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. and evaluated according to ANOVA followed by Bonferroni *t*-test. *$p<0.05$ versus PE and PEEK-WC; †$p<0.05$ versus PES, PE and PEEK-WC; ‡$p<0.05$ versus PE and PEEK-WC; §$p<0.05$ versus PSCD, PES and PEEK-WC.

Membrane	**Pore diameter [μm]**	**Porosity [%]**	**Thickness [μm]**	**Ra [nm]**	**Hydraulic permeance [L/ m^2 h mbar]**	
					native	**modified**
FC	-	-	47 ± 6.5	6.26 ± 0.91	-	-
PES	0.1	74.4±1.6	140 ± 0.5	49.38 ± 1.15	6.48 (R^2=0.99)	6.3 (R^2=0.99)
PE	0.1	79.6±3.6	23.86 ± 2.5	87.22± 1.25	126.9 (R^2= 0.98)	45.2 (R^2= 0.98)
PEEK-WC	0.2	71.2±3.1	44 ± 2.0	199.21 ± 1.05	4.0 (R^2= 0.97)	3.2 (R^2= 0.98)

Table 1 Properties of the investigated membranes.

Chapter 5

Chapter 5

Distinct $GABA_A$ α receptor subunits exert early neurogenic activities on hippocampal cells

5.1. Abstract

The modulatory actions of the major $GABA_A$ receptor subunits are known to be crucial for structural arrangement of neuronal processes. To elucidate the role of $GABA_A$ α subunits on these activities, the influences of a highly specific α_2 (flunitrazepam) agonist and of a α_5 (RY-080) selective inverse agonist on hamster hippocampal neuronal formations plus mRNA expression of NMDA receptor subunit 1 (NR1) and AMPA glutamate receptor 2 subunit (GluR2) using bio-hybrid membrane system coinstituted by hippocampal neurons and membrane were investigated. The greater effects of the two $GABA_A$ subunits occurred mostly at 7 days as indicated by reduced extension of dendritic arborization and diminished number of dendrites arising from soma in presence of α_5, while α_2 accounted for reduced axonal length and total number as early as day one. Interestingly, the α_2 agonist supplied a marked up-regulation of NR1 mRNA expression levels in neuronal processes at 3 and 7 days of development, whereas a very strong down-regulation of mainly dendritic GluR2 mRNA expression was reported after 7 days of treatment with RY-080. The trafficking ability of these two $GABA_A$ subunits is further supported by extremely low levels of BDNF throughout the entire period of culture in the presence of α_5 agonist. In addition the net up-regulatory effects of α_5 and α_2 on GluR2 and NR1 transcriptional activity, respectively, tend to propose these two major GABAergic subunits as critical elements for the recruitment and at the same time activation of silent neurons, which may constitute key sensory- and/or motor-dependent switches operating during neurogenic events.

5.2. Introduction

γ-Aminobutyric acid type A ($GABA_A$) receptors, belonging to the evolutionarily related superfamily of ligand-gated ion protein complex are regarded as major mammalian

neuroreceptor system. Of the most common 19 $GABA_A$ receptor subunits comprising the pentameric structure, α subunit appears to be the main component largely responsible for inhibitory interneuronal functions, canonical elements that are mostly GABAergic in nature [1], [2]. At present, GABAergic influences on cerebral synaptogenesis appear to be valid elements since $GABA_A$ subunits are detected earlier than glutamatergic (Gluergic) fibers during embryonic brain development [3]. However, until now neurobiological interests concerning neuronal processes have been mostly directed to the Gluergic receptor system [4] due to its maintenance of homeostatic neuronal activities [5]. Briefly the Gluergic neuronal system is composed of two main receptor classes of which the ionotropic N-methyl-D-aspartate receptor (NMDAR) and propionic acid α-amine-3 hydroxy-5 methyl-4 isoxiazode receptor (AMPAR) are involved in developmental, synaptic plasticity and excitotoxicity events [6]. It is well known that at least one NR1 plus two of NR2 (NR2A-D) are required for a functionally active NMDAR [7] while $GluR_2$ of AMPAR exerts its effects via silent $GABA_A$ neuronal mechanisms [8].

Although the culture of neuronal populations via traditional culture methods has brought us closer to the unraveling of synaptic molecular features, these methods have limited power for mimicking a physiological type of substrate, membrane systems were preferred for their well-defined micro- and nano-structures plus for their bio-stability and selective permeability properties [9]. Their use is appealing in tissue engineering since these and biomembranes share similarities such as the selective transport of molecules, resistances and protection. In the present study, the highly enriched NMDAR and AMPAR hippocampus (HIP) turns out to be a precious source of pyramidal neurons due to its highly remarkable plasticity property in cognitive tasks [10]. Hence it is our intention to evaluate the neurogenic role of $GABA_A$ α_2 and α_5 subunits on the different HIP neuronal elements of the golden hamster. These subunits, due to their synaptical and extra-synaptical localization, respectively, are critical for associative mnemonic functions as well as being dominant developmental elements of HIP [11], [12]. In addition, their regulatory role was checked to establish whether morphological

processes require NMDAR (NR1) and/or AMPAR (GluR2) subtypes plus the brain-derived neurotrophic factor (BDNF), since this factor not only modulates Gluergic and GABAergic neurosignals but also coordinates the correct membrane insertion of new AMPARs [13]. In this context, the ischemic conditions, which are typical of facultative hibernating rodents during arousal [14], makes the hamster a very useful model for studying neurodegenerative and plasticity events in the different behavioral paradigms and neurogenic processes [15]. These results could open new horizons regarding the timing of synaptic activation and consequently the induction of excitatory and inhibitory switching mechanisms during HIP developmental events in our hibernating rodent model.

5.3 Materials and Methods

5.3.1 Cell culture

The isolation and culture of HIP neurons have been optimized for a hibernating rodent model and precisely the hamster *Mesocricetus auratus* according to previous works [16], [17] plus modifications. HIP of both hemispheres was dissected from the brain of postnatal days 1-3 (PND1-3) hamsters and collected in Neurobasal medium A (Gibco, Invitrogen, Italy) containing 0.02% BSA (Sigma, Italy). The tissue was digested in a Neurobasal Medium A containing 0.1% papain and 0.02% BSA for 20 min at 37°C. Afterwards, the supernatant containing papain was removed and Neurobasal medium A supplemented with B27 (Gibco, Invitrogen, Italy), penicillin-streptomycin (100 U/ml), 0.5 mM glutamine (Biochrom AG) and 5 ng/ml β-FGF (Sigma, Italy) was added to the remaining pellet. Samples were gently triturated mechanical by using a sterile Pasteur pipette with a wide opening in order to dissociate larger aggregates. After sedimentation of these aggregates, the supernatant containing HIP cells was collected and transferred into fresh tubes containing 1% papain inhibitor in Neurobasal medium A plus 1% BSA. Hence samples were centrifuged at 1300 rpm for 10 min at RT and cell pellets were gently re-suspended in Neurobasal medium A supplement with B27, penicillin-streptomycin (100 U/ml), 0.5 mM glutamine (Biochrom AG) and 5 ng/ml β-FGF (Sigma, Italy) for plating. After isolation

procedures cell viabilities were assessed by trypan blue test, which resulted to be better (97±2%) then cells seeded on the culture surfaces at $2.5x10^5$ cell/cm^2 density. Due to the plating substrate being of great importance for survival and differentiation of neuronal cultures [18] plus traditional substrates not being highly adapt to mimic natural adhesion conditions, it was necessary, in the present study, to evaluate two different substrate systems and precisely poly-L-lysine-coated Petri dishes and Fluorocarbon (FC) membranes (In Vitro Systems & Services, Germany). The FC membranes are permeable to oxygen, carbon dioxide and aqueous vapor. Their use is particularly attractive because they allow the direct oxygenation of cells adhered to the surface. For both substrate systems, Poly-L-lysine was dissolved in a boric acid/sodium tetraborate solution (1:1) to a final concentration of 0.1 mg/ml and plated not only on Petri dishes but also on FC membranes. In particular, the latter substrate was modified by coating it with this concentration of poly-L-lysine in order to have the same functional groups over the surfaces at 40 μg/cm^2 density. The FC membranes were incubated with poly-L-lysine for 3h and then the excess of solution was removed and membranes left to dry. All culture steps were conducted in a humidified chamber under the following conditions: 5% CO_2/95% air atmosphere at 37°C for the first developmental week *in vitro*.

For this work, local government authorities approved all experimental procedures involving animals and their maintenance plus experimental procedure were in accordance with the *Guiding Principles in the Use of Animals in Toxicology*. All efforts were made to not only minimize animal suffering but also to reduce the number of specimens used.

5.3.2 Membrane characterisation

After having established morphological properties of smooth FC membranes in terms of thickness and roughness, dried membrane samples were cut and mounted with double-faced conductive adhesive tape and analyzed by scanning electron microscope (SEM; Cambridge, Stereoscan 360). From previous works cross-sectional structure, the surface morphology as well as the selective permeability to oxygen, carbon dioxide and aqueous

vapor were considered appropriate for neuronal cultures [18] and so were also considered in the present work.

5.3.3 Immunofluorescence of neuronal cell cultures

The differentiation and morphological behavior of neurons cultured on FC membranes were compared to poly-L-lysine–coated Petri dishes as controls. Representative images displaying the distribution of the neuronal cytoskeletric marker, beta-tubulin III, were obtained at DIV7 at Laser Confocal Scanning Microscopy (LCSM; Fluoview FV300, Olympus, Milan, Italy). Immunoflorescence methods, that were specific for pyramidal cell cultures, were conducted on beta-tubulin III by using primary anti-beta-tubulin III followed by secondary antibody conjugated with FICT fluorochrome on samples previously fixed and permeabilized. For this part neuronal cells that were initially fixed with paraformaldehyde (4%) for 15 min were subsequently permeabilized with 0,25% Triton X-100 in PBS for 10 min and then blocked with 1% BSA for 30 min at room temperature. The cultures were rinsed three times with PBS and incubated with the monoclonal antibody anti-beta-tubulin III (1:100; Sigma, Milan, Italy) overnight at 4°C. Afterwards, neuronal cells were rinsed with PBS and incubated with FITC conjugated antibody (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.

5.3.4 Drug treatments

To establish the role of $GABA_A$ receptor $\alpha_{2,5}$ subunits on neuronal structural formations, 100 nM and 50 nM of the highly active agonist flunitrazepam (Flu) and of the selective inverse agonist [ethyl 8-ethynyl-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4] benzodiazepine -3 carboxy-late] (RY-080), that are specific for the former and latter $GABA_A$ receptor subunits, respectively, were dissolved in 0.9% NaCl. Aliquots of these drugs were added to the culture medium immediately after isolation procedure and before plating and continuing the treatment for the first developmental week *in vitro* (DIV1, DIV3,

DIV5 up to DIV7). The selection of the two highly specific drugs was chosen on the basis of their influence on expression pattern of some $GABA_A$ receptor subunits in HIP tissue [19]. Even for this part treatments with the two agonists were handled on cells seeded with either poly-L-lysine or FC membrane and compared to culture dishes treated in the same manner only that they received a vehicle (0.9% NaCl) throughout the different DIV stages (considered controls in all experimental phases).

5.3.5 Neuronal morphology features of the two culture systems

During the entire culture period, HIP cells obtained from FC membrane were constantly monitored at an inverted microscope Axionvert 200 (Zeiss), in order to analyze the main morphological differences induced by both GABAergic selective agonists and in particular the total number of axons and dendrites, axonal and dendritic length plus soma diameter. Quantitative evaluations of these morphological parameters were determined for the numerous cell fields (100μm x 100μm) and the values of the different developmental stages, expressed as μm ± s.e.m, were analyzed by using the NIH-Scion Image software. For this part and throughout the entire treatment periods, cultured cells deriving from FC membranes were preferred, aside to the mimicking of natural adhesion conditions, this type of substrate allows a direct and accurate handling of morphometric evaluations due to the its transparent property. Moreover, preliminary morphological screenings provided similar and in some case better neuronal parameters such as the dendritic and axonal length plus cell density when FC substrates were used rather than poly-L-lysine cultures.

5.3.6 Metabolic assays

The metabolic activity of neuronal cells for the entire incubation period was determined by evaluating glucose, lactate and BDNF levels on neuronal cell medium previously collected from the two culture systems of both treated and control groups and subsequently stored in tubes at -80°C until assays. Glucose concentration in the medium was detected by using Accu-Chek Active (Roche Diagnostics, Monza Italy), while the lactate content was determined by using the lactate oxidase enzymatic assay Lactate Dry-Fast (Sentinel, Milan

Italy) via spectrophotometer analysis. The neuronal BDNF secretion was carried out on samples collected from 6 different isolations using a sensitive BDNF ELISA immunoassay (Promega Corporation WI, USA) and 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).

5.3.7 *In situ* hybridization analysis

The effects of GABAergic $\alpha_{2,5}$ agonist and inverse agonist, respectively, on Gluergic neuronal mRNA expression were determined on HIP cells at DIV3 and DIV7, since these stages are considered critical for the development of axonal and dendritic processes, respectively. The antisense and sense (nonspecific) probes specific for NR1 and GluR2 were labeled with digoxigenin-11-uridine (DIG-dUTP) by applying a terminal transferase kit (Roche Diagnostic, Milan, Italy) according to Brooks-Kayal [20], plus modifications. Neuronal cultures grown on both poly-L-lysine and FC adhesion substrates were fixed in 4% formaldehyde in PBS buffer for 15 min and then they were exposed to a 3x 1min rinse in PBS. Subsequently these cells were permeabilized in a PBS solution containing 0,2% Triton X-100 for 5 min followed by a quick rinse in PBS. The cells were next acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine and rinsed with PBS. For pre-hybridization, all cells were incubated in a hybridization buffer solution consisting of 50% formamide, 20% 5x SSC (1X SSC = 150 mM NaCl, 60mM Na-citrate, pH7.0) plus 20% dextran sulfate for 4-6 h and then 0.1-0.3 µg of probe was added to each coverslip loaded of cells and transferred to a fresh hybridization solution containing also 2% dithreitol (DTT) for an overnight incubation at 55°C. Following hybridization, cultures were washed with 2X SSC for 60 min at room temperature and then immersed for 60 min in a blocking

buffer consisting of a pH 7.5 Tris-HCl (0.1 M) + 0.15 M NaCl and 2% BSA. For immunological detections, cells were incubated with anti-DIG alkaline phosphatase antibody (Roche Diagnostic, Milan, Italy), diluted 1:1000 in the above blocking buffer for 4h at room temperature. Signals were detected according to manufacturer's instructions using the chromogenic substrates nitro blue tetrazolium (NBT; Roche Diagnostic, Milan, Italy) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Roche Diagnostic, Milan, Italy) diluted in a buffer consisting of 0.1M Tris-HCl + 0.1 M NaCl and 50mM $MgCl_2$ at pH 9.5 and cells were left in this colorimetric solution overnight at 4°C in a dark room. Afterwards these cells were rinsed for 5 min with a 100 mM Tris-HCl pH 8.00 + 1mM EDTA and then rinsed with H_2O. The coverslips were mounted using an aqueous mounting medium and observed at a bright-field Dialux EB 20 microscope. The mRNA expression capacity of NR1 or GluR2 was determined for the different neuronal portions (axon, dendrites and soma) in the presence of both Flu and RY-080 and compared to controls via the application of a Macintosh computer-assisted image analyzer system running Image software of National Institute of Health (Scion-Image 2.0) plus a constructed internal standard curve for calibrating optical density (O.D.) values.

5.3.8 Statistical analysis

The quantitative morphometric (μm ± s.e.m.) differences among the different neuronal processes (axon, dendrite and soma), induced by the two α GABAA subunits at different developmental stages (DIV1, DIV3, DIV5 and DIV7) plus *in situ* hybridization values referred to DIV3 and DIV7 and reported as means of O.D. ± s.e.m., were compared by a two-tail ANOVA followed by Neuman Keul's Multiple Range Test when $p<0.05$. For metabolic assays, the statistical significance was established according to the ANOVA test followed by unpaired Statistical Student's *t*-test ($p < 0.05$).

5.4 Results

5.4.1 Morphometric evaluations of neuronal processes from HIP culture

For the present study it was our intention to investigate the experimental validity of bio-hybrid membrane system constituted of FC membrane and hippocampal neurons to study the different pharmacological effects on HIP neuronal formations of the hamster. From the early developmental stages (DIV3), it was not possible to detect any substantial growth differences of HIP neuronal populations plated on either FC membrane (Fig. 1a) or poly-L-lysine substrate (Fig. 1d) as demonstrated by similar dendritic and axonal lengths (Fig. 1g, h) during this growth period. On the other hand, the former plating substrate rather than that of poly-L-lysine began to exhibit favorable conditions at a later developmental period (DIV7) as shown by the highly enriched cultured areas containing the various neuronal elements (Fig. 1b,e) despite no differences of soma diameter (Fig. 1i). In these cultured areas it was possible to observe moderately (53%, $p<0.05$) and highly significant (82%, $p<0.01$) increases of dendritic and axonal lengths, respectively, as well as above all, a constantly high level of total cell density (approximately 2.56×10^5 cells/cm^2) on both DIV3 and DIV7 days of growth (Fig. 1j). The favorable growth stages coinciding with the latter was further supported (at the LCSM) by an intense, but at the same time heterogeneous expression pattern of β-tubulin III (specific for dendritic and axonal elements), which underlies a widespread density of synaptic in both culture systems (Fig. 1 c,f).

5.4.2 Role of GABA$_A$ α subunits on neuronal metabolism and morphological features

From the determination of some metabolic parameters and namely glucose consumption, lactate production and BDNF release it seems that these parameters did not modify the growth rates of neither FC nor poly-L-lysine substrates (Fig. 2a,b) until the drugs of the two GABA$_A$ α subunits were not added. For this reason and on the basis of the above evidences regarding better culture conditions supplied by FC membranes, only the latter membrane culture system was applied to evaluate the role played by the two GABA$_A$ α subunits on neuronal metabolism and morphological features. In a first case, the addition

of the α_5 inverse agonist (RY-080) seemed to be responsible for the major effects. This relationship was supported on the one hand from the highly significant reduction (-83%) of BDNF levels (Fig. 2c) plus the moderately significant increase of glucose consumption (+43%) and lactate production (+45%) in the presence of RY-080 at DIV7 (Fig. 2a,b). On the other hand a moderately significant increase of glucose consumption (+32%) was detected when the cells were treated this time with the specific α_2 subunit agonist (Flu) at this same developmental stage (Fig. 2a).

The effects of these two functionally important $GABA_A$ subunits were also investigated to check their role on the different neuronal formations. In this case, it appeared that both subunits behaved in a somewhat peculiar manner towards the morphological shaping of the neuronal elements. Regarding the extra-synaptical subunit (α_5), it was mostly responsible for an evident reduction of the dendritic arborization length (Fig. 3a) with respect to control cells (Fig. 3b). Surprisingly this effect resulted to be even greater when these values, expressed as a ratio with respect to their controls (check figure inserts), were compared among the two subunits. Indeed a very robust ($p<0.001$) reduction was detected at DIV5 and DIV7 when compared to α_2 subunit (Fig. 3c). At the same time, the effects of α_5 was also responsible for a very robust decrease of the total number of dendrites arising from soma with respect to the other GABAergic subunit (Fig. 3d) during these developmental stages while the agonist of the synaptical subunit α_2 only supplied a very robust reduction of dendritic length at DIV1 (Fig. 3c). The effects of the GABAergic α_2 subunit seemed to be, however, preferentially directed towards the axon (Fig. 4a) than control cells (Fig.4b), especially during the early developmental stages as demonstrated by the very robust reduction of axonal length (Fig. 4c) at DIV1 and DIV3 with respect to α_5. In this same growth period the effects of the α_2 subunit agonist also accounted for a very robust reduction of the total number of axons when compared to the other GABAergic subunit (Fig. 4d). The effect of this subunit tends to, however, decline as time passes on as shown by moderately significant decreased axonal lengths at DIV5 and DIV7 (Fig. 4c).

5.4.3 Effects of $\alpha_{2,5}$ subunits on Gluergic receptor mRNA expression levels

The effect of the GABAergic agonist and inverse agonist, specific for $\alpha_{2,5}$ subunits, respectively, were next evaluated on Gluergic neuronal mRNA expression capacities in HIP cells at DIV3 and DIV7. A first observation, considered of up most value, is that FC substrate did not reveal any background non-specific noises outside of the cells as reported when expression capacities were tested on poly-L-lysine substrate (Fig. 5a,b). Surprisingly, it was still α_5 that induced greatly significant variations even though they occurred at DIV7 (Fig. 5c) as shown by the very robust and highly significant down-regulatory effects of GluR2 transcript in dendrites (-108%) and axons (-75%), respectively (Fig. 5e), than at DIV3 following treatment with RY-080 (Fig. 5d).

In the case of the specific α_2 subunit agonist, it specifically induced changes of NR1 mRNA expression activities as revealed again by the similar expression activities of FC membranes and of poly-L-lysine substrate at DIV3 (Fig. 6a-c). Indeed, this subunit was mostly involved with a highly consistent expression capacity of NR1 transcript at the axonal level (+78%) while a moderate increase characterized the neuronal soma (+48%, Fig. 6d). Interestingly, although the former neuronal element continued to be the major target of Flu as supported by the highly significant up-regulation of NR1 mRNA densities at DIV7 (+82%), it was not the only element since dendrites also displayed a similar effect, as demonstrated by the moderate increase of NR1 expression levels at the same developmental stage (Fig. 6e).

5.5 Discussion

The results of the present study highlight the importance of FC membrane biohybrid system for the determination of $\alpha_{2,5}$ GABAergic and Gluergic subunits responsible for the successful elongation processes of neuronal elements in hamster HIP cells. Up to date the different synaptic properties along with their organization and plasticity mechanisms have been extensively studied on neurons grown on the traditional type of substrates [21] while FC membranes are beginning to offer greater advantages for the *in vitro* evaluation of

neuronal circuits as a key physiological implant for neural tissue engineering [22] .These first morphological successes of HIP cells grown on FC substrates with respect to poly-L-lysine substrate confirmed the well-developed and above all integral topographical characteristics of the different neuronal elements such as a dense number plus greater dimension of axons and dendrites grown under different culture conditions [23] in our rodent model. Even the elevated levels of cytoskeletal marker such as beta-tubulin III and of some main metabolic parameters (glucose, lactate and BDNF), which are similar to those obtained using poly-L-lysine substrate, tend to further support the value of this type of culture substrate [24]. As a consequence, the widely differentiated morphology of large pyramidal soma along with the sharp branching processes could very well represent an important condition in order to define *in vivo* plasticity and functional features of mnemonic and learning paradigms that are typical of HIP.

Our attention next shifted to the specific structural morphological and transcriptional expression roles of the two α GABAergic subunits ($\alpha_{2,5}$) of HIP neurons. A number of works have reported that α subunits display considerable kinetic and pharmacological diversities during the inhibitory propagating activities, which are tightly related to the cerebral regional-and domain-specific localization of the six different α subunits. The fact that $\alpha_{2,5}$ are the most common HIP subunits responsible for the numerously distinct functional properties such as post-natal developmental plasticity, sleep, memory and sensorimotor activities [25], [26] strengthen their active neurogenic role during the formation of the neuronal elements of this brain area. Indeed, the differentiated shaping and elongating processes occurring on DIV 1 in the presence of both subunits seem to immediately underlie their early neurogenic-dependent activity. Interestingly, when the culture system was treated with the highly specific agonist for α_2, it mainly accounted for both a reduced number of total axons and overall length during the early developmental stages. This type of relationship seems to fit nicely with the initial axonal segments plus synaptogenic processes of HIP pyramidal cells being strongly linked with the formation of immature GABAergic synapses containing clusters of $GABA_A$ α_2 subunits [27] which

suggests a critical role of such a subunit during the onset of neuronal polarization, extension of neuritis and early connections of complex neuronal networks. It is tempting to suggest that the innervation mechanisms operating during the early neurogenic events of HIP cells appears to be predominantly guided by an excitatory α_2 type of interneuronal activity [28], very likely via its binding to gephyrin [29]. The plasticity of α_2, however, is not only restricted to the early neuronal elongating processes because at a later stage contemporary to the reduced number of α_2-enriched $GABA_A$ sites, there is also a prevalent functional turnover as shown by the major HIP activities being mostly assumed by the greater densities of α_1 subunits [30]. In this case the dense concentration of α_2 in the ventral part of HIP [31] seems to be favorably involved in temporal memory and spatial recognition processes [32], probably through cross-talking mechanisms with the same $GABA_A$ subunits of another major limbic areas and precisely the amygdale [33]. The role of the GABAergic neuroreceptor system appears to be also involved with other critical neurogenic developmental stages, occurring at different moments as shown by α_5 subunit achieving structural variations at >DIV3. As a matter of fact when the selective inverse agonist RY-080, specific for the extrasynaptic α_5 subunit was added, it turned out to be a major determinant factor involved in the blocking of GABAergic activities and consequently reduced the number of branching elements and dendritic length at DIV7. These results are in good agreement with the early development of spine heads and dendritic arborization occurring in a nonsynaptic $GABA_A$ receptor-dependent manner [34]. On the basis of such structural features proceeding along the same developmental pattern as those involving members of the scaffold proteins and namely Shank-1 [35], it would seem reasonable to propose the specific interaction of the main extrasynaptic $GABA_A$ subunit α_5 together with Shank-1, as a principal neuronal growth factor for the construction of GABAergic synapses and eventually the dynamic modulation of inhibitory pathways [36].

In addition the morphological variations induced by α_5 tend to show a very strong BDNF-dependent type of relationship. Indeed during the later developmental stage, which seems

to be the most favorable period for the effects of this subunit, decreased BDNF levels were detected concomitantly to the reduced dendritic patterns in the presence of the α_5 subunit selective inverse agonist. The effects of this neurotrophic factor with specific cytoskeletal actions could very well be responsible for pre-synaptic dendritic arborization of not only adult but above all of embryonic HIP neurons [37] as well as fast-spiking GABAergic actions [38] that are typical of only inhibitory cortical pyramidal neurons [39]. Consequently, the predominating BDNF role on α_5 subunit processes appears to be strongly consistent with a GABAergic neuronal switch for both morphological and functional features [40]. Surprisingly, the central role played by α_5 during neuronal development continues to be also conserved throughout the later biological period in which the onset of inhibitory mechanisms in the different HIP areas are facilitated via α_5 $GABA_A$ receptors especially during the acquisition and expression of associative memory, as well as pre-pulse inhibition of locomotor exploration and cognitive functions [41].

The results of the present study plus those of other works corroborate structural formations and above all the consolidation of complex neuronal networks heavily requiring specific timing on/off switches that are regulated by cross-talking mechanisms of GABAergic (inhibitory) and Gluergic (excitatory) receptor subunits [42] during the different developmental stages [43]. Working along the lines of these major excitatory and inhibitory homeostatic states, which control to a large extent most neurophysiological and behavioral functions, the synergic interaction of both neuroreceptor systems might unravel intriguing neuronal signals regarding the embryonic formation of neuronal processes. The fact that the $GABA_A$ α_5 subunit preferentially controls the transcription of the Gluergic site (GluR2) involved in silent neuron activity brings us closer to the determination of a probable mechanism operating on dendritic arborization structures. Indeed the elevated levels of this Gluergic subtype induced by α_5, in a BDNF-related manner, as observed from RY-080 being responsible for the reduction of GluR2 at DIV7, suggest that the later neuronal formations also require GluR2. As a consequence, the participation of GluR2 might be of the retrieval type of effect since this Gluergic subunit is capable of re-

establishing contacts with other neuronal systems as displayed in AMPAR2 devoid areas and so in turn an α_5-induced upregulation of GluR2 could very well improve the trafficking strategies of extrasynaptic spine and dendritic elongating processes as already reported during plasticity events of rat HIP at DIV14-21 [44]. In this case, the lack of residual tonic current promoted by RY-080 during the early developmental stages may not only cause the incorrect formation of HIP interneurons but furthermore might very well compromise the adequate assembly of GABAergic and Gluergic terminals [45] and subsequently the mediation of a lion's share of tonic inhibition of HIP CA1 fields [46]. Moreover, the importance of α_5-linked GluR2 on neuronal processes is provided not only at the functional state by GluR2-containing clusters being incorporated at NR1 sites and so capable of activating silent neurons [47], but also at the structural level through the abnormal morphology of dendritic spines and synapses complexes[48].

Interestingly, with the addition of the α_2 subunit agonist, a marked increase of axonal NR1 sites was mostly detected during DIV3. This result tends to confirm the primary role of α_2 on the early neuronal developmental stages of rat very likely via the facilitation of a NMDAR-dependent enhancement of NR1-synaptophysin complex, which is linked to the maintenance of neurons in a silent state [49]. It is only at a later stage that these silent neurons appear to be activated via the clustering of the α_5-dependent GluR2 production plus the dense number of NR1 sites (Voigt et al., 2005). The important role of α_2 is further supported by successful neurite elongating processes of rat HIP neurons [50]. Even though at a later state and this should not surprise us because the HIP of hamster, used in the present study, are known for its highly rich reservoir of factors ready for dendritic formations at a much earlier time [51]. Contemporarily, the up-regulatory effect of α_2 on NR1 appears to induce a correct assembly of NMDAR through the initial formation of NR1/NR2 and NR1/NR3 heterodimers and thus constituting the first step towards a morpho-functionally stable NMDAR complex [52]. Once this stability is achieved, NR1 can next interact with specific synaptic adhesion-like molecules (SALMs) modulating, aside neurite outgrowth, also the activation of dendritic cytoskeletal dynamics, synaptic

formations [53] and thereby providing adequate conditions for subsequent developmental processes.

Overall, these first results dealing with the GABAergic effects on hamster HIP neuronal elongating processes using the highly efficient FC membranes for neural tissue engineering [54] allowed us to underlie the major developmental role played by the GABA$_A$ $\alpha_{2,5}$ subunits through a Gluergic mechanism. In particular during the first days of culture activity, considered a delicate interval for the success of the different neuronal elements, the tight interaction between GABAergic ($\alpha_{2,5}$) and Gluergic (NR1/GluR2) systems may constitute an early but at the same pertinent element that is crucial for regulating axonal sprouting, dendritic protusion motility [55] and above all the early activation of silent synapses [56]. In this context, the highlighting of the neuronal developmental processes guided by these two major inhibitory and excitatory circuits propose the novel molecular and synaptic plasticity mechanisms influencing HIP genomic neuronal programs of the hamster that may prove to be critical above all during some stages of hibernation [57] and precisely torpor and arousal.

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List of symbols

$GABA_A$ = γ-Aminobutyric acid type A

NMDAR = N-methyl-D-aspartate receptor

AMPAR = α-amine-3 hydroxy-5 methyl-4 isoxiazode receptor

HIP = Hippocampus

BDNF = Brain-derived neuronal factor

b-FGF = Basic fibroblast Growth Factor

BSA = Bovine Serum Albumin

DAPI = 4'-6-Diamidino-2-phenylindole

DIV = Day in vivo

ELISA = Enzyme-Linked ImmunoSorbent Assay

FC = Fluorocarbon

FITC =Fluorescein isothyocianate

Flu = Flunitrazepam

HCl = Hydrochloric acid

PBS = Phospate-Buffered Saline

NaCl =Sodium Chloride

RY-080 = [ethyl 8-ethynyl-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4] benzodiazepine -3 carboxy-late]

SEM = Scanning Electron Microscope

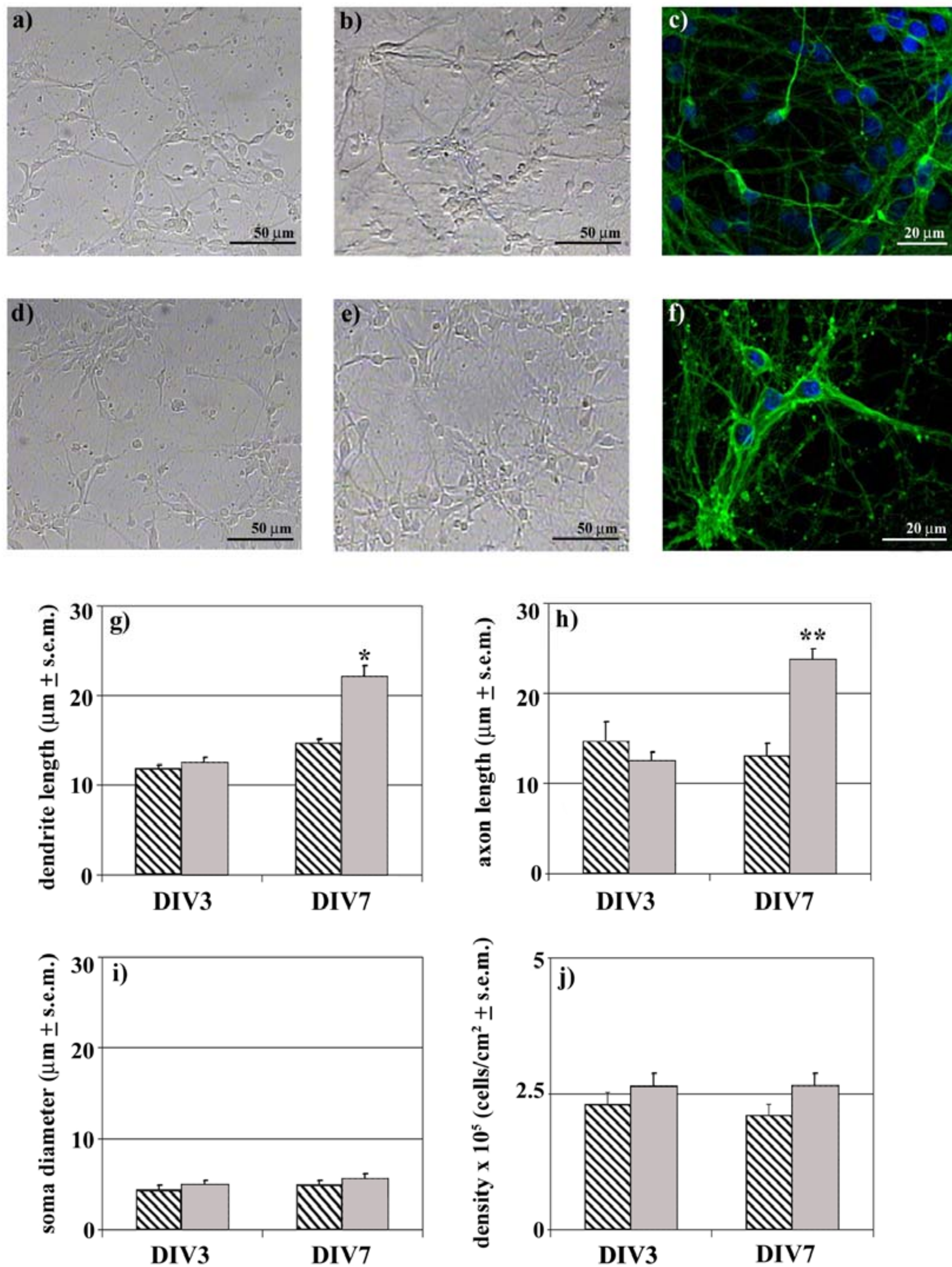


Fig. 1

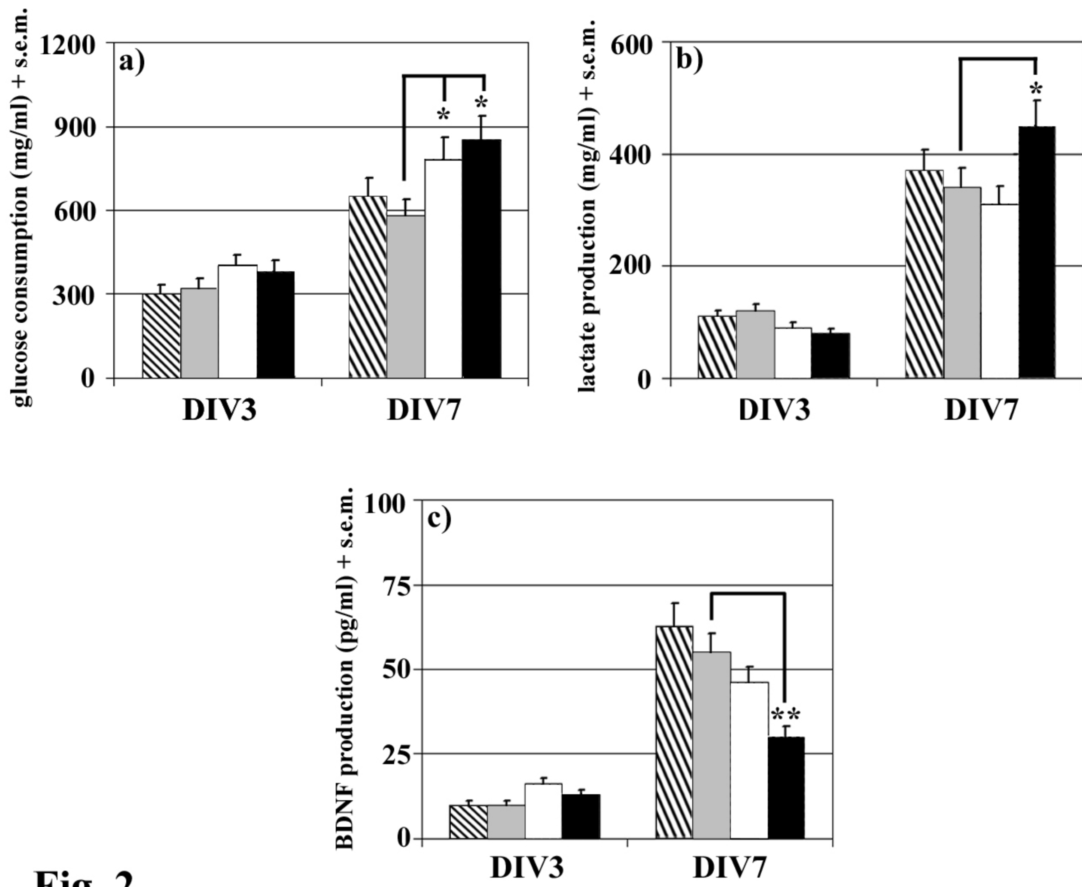


Fig. 2

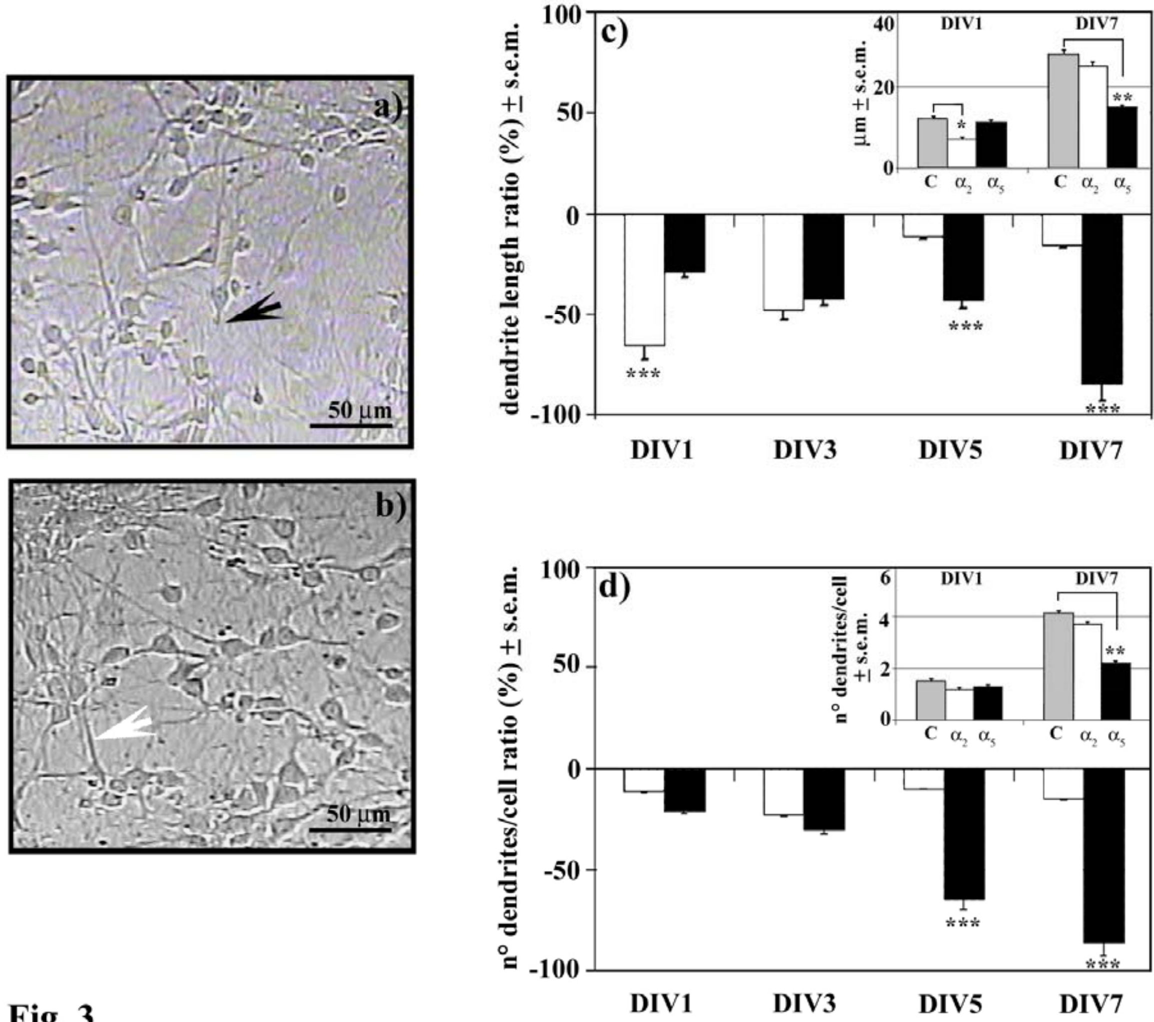
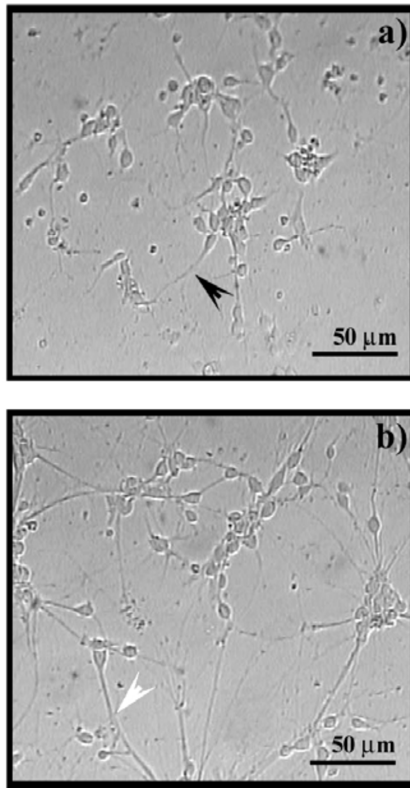


Fig. 3



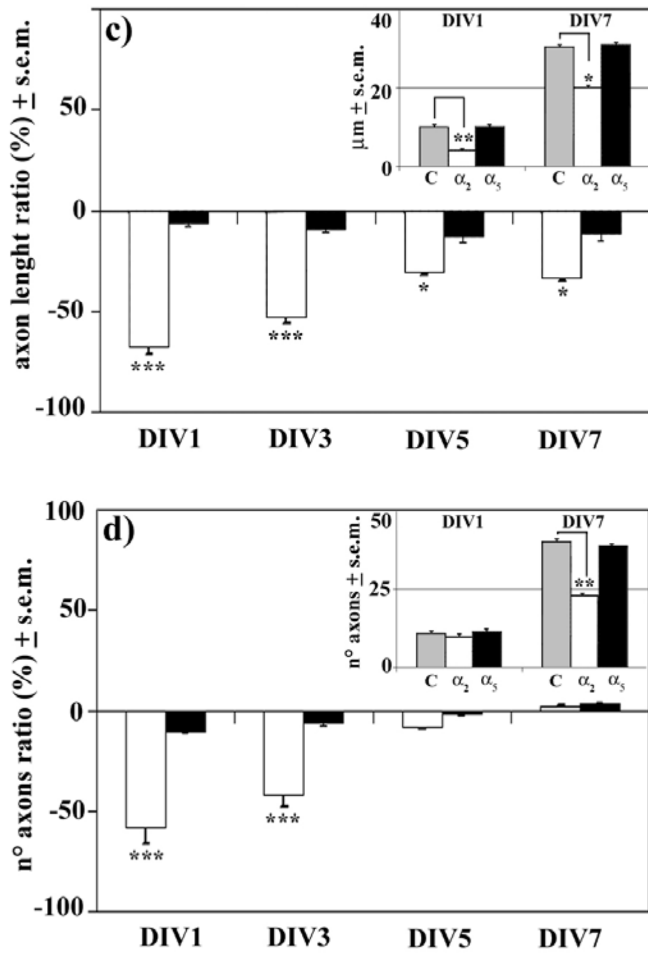


Fig. 4

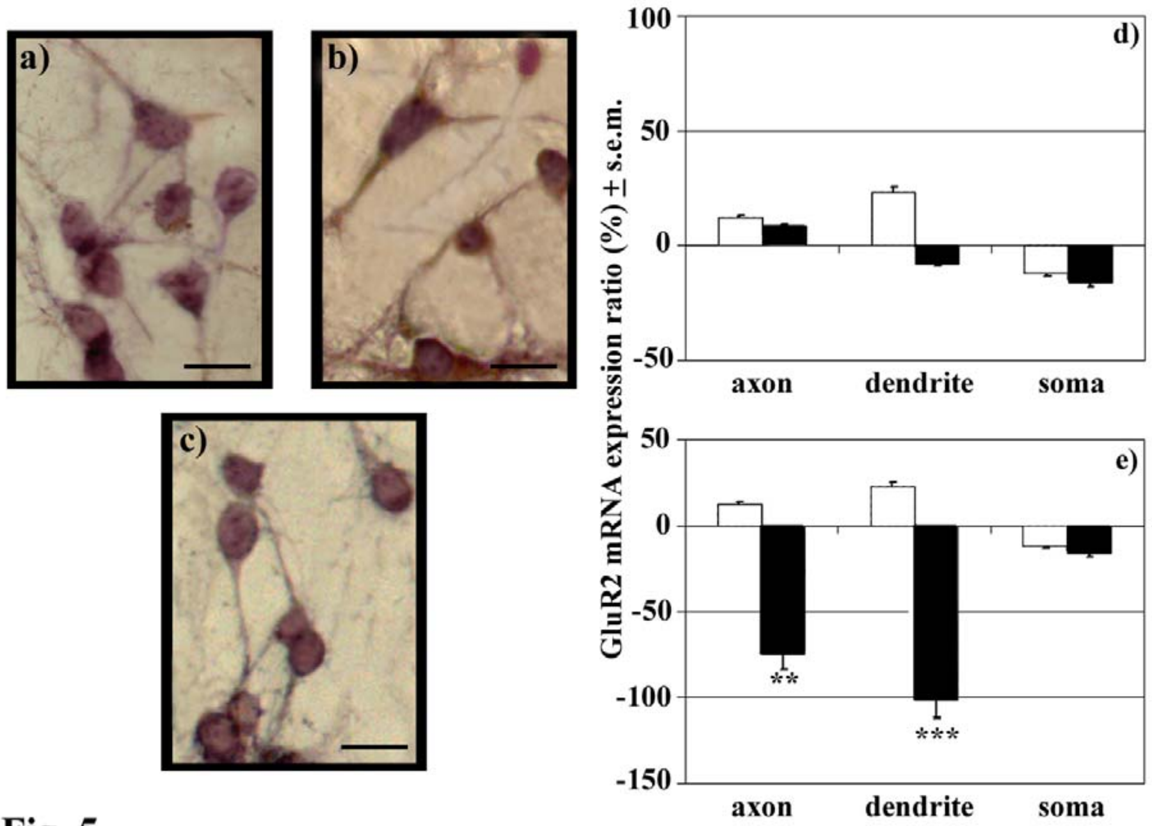


Fig. 5

Chapter 6

Chapter 6

Hollow fibers membrane for culture of hippocampal cells

6.1 Abstract

Tissue engineering applied to the central nervous system (CNS) is an emerging field which promise developing of new biomaterials for supporting the growth of neuronal cells. Central nervous system neurons do not regenerate spontaneously following the injury in nervous damage. Membranes can be used in neuronal implant applications for the treatment of diseases such Parkinson's, or Alzheimer's or *in vitro* system to reproduce brain functions to boned for drug testing toxicology.

Polyetheretherketone (PEEK-WC) and Polyacrilonitrile (PAN) hollow fibers microporous membranes (HFMs) were prepared for supporting the growth of hippocampal neuronal cells. The polymeric substrates were evaluated for their ability to control hippocampal neuronal cell adhesion and direct axon regeneration.

Neurons isolated from the hippocampus of *mesocricetus auratus* were cultured outside the HFMs which ware previously coated with poly-L-lysine (PLL) in order to favour cell adhesion and growth. The membranes structure and their relationships with cells were examined by scanning electron microscopy. The behaviour of the cells was tested through evaluation of morphology and neurites elongation and metabolic functions at different day's culture. Results demonstrate that a high percentage of cells were found to be attached to PEEK-WC–HFMs and PAN-HFMs, produced a large number of neurites processes, which organized in a very complex network.

Cells are metabolically active as is demonstrated not only, by to glucose consumption and lactate production, but also by the secretion of brain-derived neurotrophic factor (BDNF).

Results provided the first evidence of the hippocampal cells adhered and growth on HFMs. These membranes for the selective transport properties of nutrients and metabolites can be used in future for prepare a membrane bioreactor to maintain cell viability and functions *in vitro*.

6.2 Introduction

Membrane in hollow fiber configurations are particularly advantageous in the application of neuronal regeneration owing their tubular architecture that gives a topographical guidance to the regenerating axons [1]. In fact is well known that a loss of such architecture results in the disorganization of the axons, even those that survive the injury. Therefore synthetic hollow fiber membranes can be used to repair the nerve transaction. Transplantation of porous tubes in membranes in poly(2hydroxyethylmethacrylate-co-methyl methacrylate) was performed by Reynolds et. Al, following a spinal cord transaction surgery in order to improve locomotor functions in rat [2].

Hollow fiber 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 antibodies and complement components from entering the membrane lumen and interacting directly with the encapsulated cells [3]. One of the application of hollow fiber membranes with encapsulated cells regards the implantation in the CNS for treatment of Parkinson’s disease. Parkinson’s disease is a neurologic disease characterised by the progressive loss of dopaminergic neurons in the substancia nigra pars compacta, wich are important to motor control.

In my study hollow fiber membranes are used to develop an *in vitro* functional biohybrid system with hippocampal neurons. Hollow fiber membranes offer to the cells: i) a wide surface area for the adhesion and for the mass exchange in a small volume, ii) the compartmentalization in a well defined microstructure, iii) perfusion without shear stress.

The molecular weight can be adjusted to allow diffusion of nutrient, waste products, and the external humoral factors that are beneficial to regeneration across the membrane tissue interface, but exclude the transport of large immunogeneic molecules.

Hollow fiber membranes can be used to realize *in vitro* a tissue engineered construct in which neuronal cells are continuously perfused with nutrients and metabolites.

On the basis of the results obtained from previous study on the effect of morphological properties of membranes surfaces on neuronal growth, were designed HFMs with low porous external surface.

To this purpose all parameters influencing the morphology of the HFMs such as concentration of polymer, type of solvents and additives, composition of coagulation bath, temperature of spinning and coagulation bath were identified.

For the preparation of HFMs two biocompatible polymers of modified polietheretherketone (PEEK-WC) and polyacrilonitrile (PAN) membranes were chosen [4]. PEEK-WC-HFMs has been used for the culture of other type of cells such as human lymphocytes in a bioreactor; De Bartolo et al. demonstrated that PEEK-WC HFMs ensure sufficient transport of metabolites and nutrients for the maintenance of cell viability and functions [4]. These HFMs were coated with poly-L-lysine (PLL), in order to favour the adhesion cells being the substrate that allows the *in vitro* growth of neuronal cells [6].

The morphological and transport properties of the membranes were characterized before to use them for cell culture.

Neuronal growth and metabolic behaviour of cells were compared between PEEK-WC and PAN HFMs which have different morphological and transport properties.

Metabolic activities of the neuronal cells were monitored with respect to glucose consumption and lactate production. The secretion of Brain-derived neuronal factor (BDNF) secretion was used as marker of the functional development hippocampal tissue, since this neurotrophic factor with specific cytoskeletal functions appeared to be also responsible for pre-synaptic dendritic arborization of embryonic hippocampal neurons [7].

6.3 Materials and Methods

6.3.1 HFMs

Membranes were prepared from modified polyetheretherketone (PEEK-WC-HFMs) 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 PAN polyacrilonitrile, according to the well-know dry-wet spinning method [8]. PEEK-WC-HFMs membranes were prepared from 18 % (wt/wt) PEEK-WC polymer, in dimethylamide (DMA), whereas PAN-HFMs membranes were prepared from 15 % (wt/wt) PAN and 15% (wt/wt) PEG (w/w) in dimethylformamide (DMF).

Solutions of PEEK-WC and PAN were prepared by slow addition of the polymers to the solvent under continuous mechanical stirring at room temperature. The polymers were previously dried overnight at 70°C under vacuum. The solution was stirred for three hours and then left standing for three hours to remove air bubbles.

The polymer solution (dope) was filtered on a 15 micron stainless steel filter and then loaded into the dope vessel kept at 30°C. The dope was fed to a coaxial tube spinneret by an N_2 overpressure and the flow rate was checked gravimetrically. The bore solutions casting of a mixture of DMA and water at 60 and 40 wt.% respectively for PEEK-WC-HFMs and a mixture of DMF and water at 60 and 40 wt.%, it were fed to the spinneret with 0.8 mm hole diameter and a needle for the bore fluid with in external diameter of 0.4 mm. The polymer solution leaving the spinneret entered an air gap which was adjusted to 60 cm before dipping into the coagulation bath. In this rotating water bath the fibres were collected at the bottom as a continuous filament at the end of the spinning test.

The continuous filament was then cut in pieces of about 30 cm and kept in a pure water bath for 48 h by replacing water two times a day to remove residual solvent and the addictive for PAN-HFMs membranes. The membranes were soaked in a 20 wt % aqueous glycerol solution for 24 hours and finally dried at room temperature for at least 48 h [4].

The 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 $\mu g/cm^2$. 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.

6.3.2 Membrane characterisation

Dried PEEK-WC-HFMs and PAN-HFMs membrane samples were cut in cross-section, mounted with double-faced conductive adhesive tape and analyzed by scanning electron microscope (Quanta 200F ESEM, FEI, USA). From some selected representative images it was possible to observe the typical morphology of the membranes and obtain information about cross-sectional structure and thickness, intra and extra-lumen morphology and diameters (structural properties are reported in Table 1).

The cut-off of the PAN and PEEK-WC membranes was evaluated by permeability measurements by using dextranes with different molecular weight (10500, 66700, 81000 Da). The rejection of the membrane was defined on the basis of the following equation:

$$R = 1 - (Cp/Cf)$$

The hydraulic permeance of the membranes was evaluated by pure water flux measurements in the absence of solutes and at different transmembrane pressure (ΔP^{TM}). For each membrane the hydraulic permeance L_p, was evaluated before and after modification process with PLL by the following equation [9]:

$$L_P = \left(\frac{J_{Solvent}}{\Delta P^{TM}} \right)_{\Delta c = 0}$$

This equation assumes a linear correlation between water flux and the convective driving force.

6.3.3 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 [6]. 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 [5]. 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 [6, 10, 11]. 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.

6.3.4 Immunocytochemical markers 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 4 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 anti- βIII-tubulin followed by secondary antibody conjugated with FITC fluorochrome on samples previously fixed and permeabilized [9,12]. 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 anti- 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.

6.3.6 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 4, 8 and 12 days *in vitro.*

6.3.7 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 ($p < 0.05$).

6.4 Results

HFMs membranes displayed a different morphology as Fig. 2-3 shows. PEEK-WC-HFMs are characterized from a dense skin layer with pores with an elongated shape, and a matrix inside with fingers pore (Fig.2). PAN-HFMs have a structure with homogeneous pore distribution, without skin layer (Fig.3). The morphological properties of both membranes PEEK-WC and PAN membranes have a wall thicknes of 135 ± 5μm, 104.2 ± 7μm respectively and internal diameter of 562.5 ± 9.6 μm and 683 ± 7.2 μm respctively and external diameter of 833 ± 5.8 μm and 891.7 ± 7.2μm (Table 1).

The cut-off values of the PEEK-WC and PAN HFMs were ~78000 Da and ~81000 Da respectively (Tab.1). This involve that molecules with molecular weight greater than 78000 Da are rejected from the PEEK-WC HFMs while PAN HFMs retain molecules with molecular weight greater than 81000 Da.

The membranes exhibited also different permeability properties (Fig.4). Hydraulic permeance of the investigated membranes was calculated accordingly as the slope of the flux J versus the transmembrane pressure (ΔP^{TM}) straight line. The convective metabolites transport through HFMs depends on the membrane hydraulic permeance Lp, evaluated from filtration flux in the absence of solutes and at different transmembrane pressure. PAN membrane was more permeable with respect to PEEK-WC membranes. This depends to small value of membrane thickness and different pores size and distributions.

After characterization of morphological and permeability properties, the membranes were used for hippocampal cell culture. The viability of cells after the isolation from hippocampus was 98 % (Tab.1).

Hippocampal neurons adhere at high density to the both surfaces and developing cellular processes already during the first days of culture. (Figs.5a and 6a). With the progress of their growth period (Figs. 5b and 6b), dendrites and axons subsequently the formation of synaptic contacts developed into a rich neuronal network (Figs. 5c and 6c). The complexity of the neuronal network increased with time: dendrites became highly branched and adapted at the topographical surfaces of the membranes (Figs. 5d and 6d).

The neuronal network on PEEK-WC-HFMs is quite homogeneous and densely distributed but on PAN-HFMs, the cells adhered forming several compartments witch develop neuronal processes highly branched (Figs. 5c and 6c).

Consistently with the morphological analysis neurons on PEEK-WC HFMs and on control substrate (PSCD) significantly longer than those PAN-HFMs. The length of neuronal processes increased with time (Fig. 7).

The metabolic activity of cells support the hypothesis that HFMs have a good biocompatibility. Glucose consumption and lactate production were exhibited at similar levels on PEEK-WC and PAN HFMs for the most of culture time (Figs. 8a, b). An exception was noticed at day 12: cells on PAN shown a greater consumption of glucose and production of lactate with respect to other substarate. This demonstrates that hippocampal cells adapt very well at new membrane configuration.

BDNF secretion was expressed by cells on different substrate at similar level. Also for BDNF secretion at day 12 significant higher activity of cells was measured on PAN-HF. This support the conservation of vital cell function during the growth periods (Fig. 8c). The evaluation of the metabolic activity showed that cells maintain their function in terms of BDNF secretion at different levels throughout the entire culture period.

6.5 Discussion

The adult central nervous system (CNS) is not capable of regenerate; regeneration is limited by the fact that adult neurons are not capable of proliferating.

Since many factors play roles in affecting CNS regeneration, it is unlikely that any single strategy will completely reverse the consequences of adult CNS injury. Conventional strategies that are centred on the uses of either biomaterials or cells alone have be inadequate to elicit a significant regenerative response from severed CNS axons of mature nervous system; as a result, approaches utilizing tissue-engineering principles have been pursued. A number of engineered substrates containing oriented ECM, cells or channels have displayed potential of supporting axonal regeneration and functional recovery [12].

In this work we have focused on the development of HFM, used for 3D-tissue engineered construct using primary hippocampal neurons as cells source.

The membranes were modified by coating with PLL in order to minimize their native different physico-chemical properties and to have the same functional groups interacting with cells. The high hydraulic permeance of HFMs after coating provide a sufficient level of mass transfer efficiency to ensure the survival of neuronal cells and efficient mass transfer of oxygen, nutrients and metabolites.

Neuronal cells on the membranes displayed the morphology that included a primary apical dendrite with multiple ramifications, very thin axons plus a discretely flattened soma.

Neuronal cells adhere outside the HF membrane surface and develop a typical well-definite shape of the hippocampal neurons, with a primary apical axon and many branched dentrites. With subsequent development, the density of the axonal network increases, dentritic arbors become more elaborate and highly branched, synaptic contacts develop in a large number propagating the neuronal network. The density of axonal network increases with the time and the neurites become more elaborate and highly branched on the smooth surfaces. Neurites outgrowth from cell body depends of the topographical characteristics of the surface. On PEEK-WC-HFMs membranes, the neurons were developed in a organized monolayer, perfectly distributed on the surface. On PAN-HFMs membranes cells tend to aggregate in a different compartments and neurites outgrowth exhibits shorter processes.

These data are consistent with the morphological behaviour of cells that tend to form several areas of aggregation that become points from which neuronal network develop. In this cell organization, neuronal processes are shortened owing there more close contact points with the neighbouring cells.
Probably the more porous surface of PAN HFMs influence cell distribution, extension and ramification of neuronal processes, and the adsorption of adhesion proteins necessary for the interaction with membrane surfaces.
In this case we are obtained hippocampal cells growth on 3D semipermeable membranes, with different permeability, that could be used in a dynamic cell culture.
The confocal images show the microtubule organization typical of mature hippocampal cells. βIII-tubulin (green) is the major cytoskeletal markers, which is specific for neuronal processes and soma and GAP-43 (red) is membrane protein associated to axon outgrowth. Both this marker emphasizes the hippocampal neurons development. [13].
This result confirms the capability of the HF membrane to promote the neuronal networks for a greater period of time more than that supplied by other substrates (Fig.9a, b) [14]. This constant expression of βIII-tubulin turns out to be very important since like others have reported cytoskeletal proteins are involved in basic cellular activities that include cell–cell interaction, cell adhesion and migration and any eventual damage may lead to the total degradation of neuronal processes [5].
The metabolic activity of neurons on membranes demonstrated that cells adhered on the membranes are functionally active for 12 days of culture. Glucose consumption and BDNF secretion were measured for 12 days of culture, the results show are not statistical differences between the investigated membranes and to respect the control substrate (Fig.11a). The glucose consumption is constant for the entire culture period that demonstrate that the cells adhering to the HFMs are also functionally active.
The lactate production rate was lower in the PEEK-WC-HFMs which respect to the PAN-HFMs and the PDSC; this suggests a shift of aerobic metabolism of the neuronal cells on PEEK-WC-HFMs.

The BDNF is a mammalian neurotrophin that have an important role in the survival, differentiation and maintenance of specific neuronal populations. This neurotrophin have an important role too in the synaptic plasticity and in the memory formation. Low protein's secretion is associated to a diverse disease states, including Alzheimer's disease, depression, pain and asthma [15]. Hippocampal neurons exhibited high levels of BDNF secretion on HFMs where cells formed highly branched neurites and a more complex network. The constant levels of secretion, confirm the viable status of hippocampal neurons on HFMs throughout the culture period. Elevated productions of such a cellular protein at high level at 12 days of culture demonstrate that synaptic transmissions communicate between the hippocampal cells in the intricate neuronal network (Fig.11c) [16]. Moreover this neurotrophin is distributed not only along dendrites and therefore located postsynaptically but also presynaptically along the axon [17].

6.6 Conclusions

This study reports the neuronal tissue regeneration on PEEK-WC-HFMs and PAN-HFMs as potential substrate in membrane bioreactor. Hippocampal neurons isolated from hippocampus are cultured outside this membrane in a hollow fiber bio-hybrid system. Neuronal cells respond to this topographic surface with morphology and neurite outgrowth, similar to the *in vivo* tissue. Cells develop cellular processes and of length compare to the natural support (poly-L-lysine). Glucose consumption displays the active cellular metabolism of cells; the elevated secretion of BDNF indicates completely maturation of hippocampal cells (8 days) membrane surface up to. Results of this study encourage the development of a membrane bioreactor for hippocampal neurons growth, which is able to regenerate neural tissue in a well-controlled microenvironment and tested the effects of develop neuronal network in presence of physical and chemical stimuli too. Use of PEEK-WC-HFMs and PAN-HFMs, characterised to a different permeability, could be important elements for the application in a membrane bioreactor for in vitro neuronal tissue engineering investigation or in vivo environment . The membrane bioreactor could be

potential valuable tools aimed to study and understand cellular mechanisms of neurodegenerative processes and for drug testing.

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List of symbols

BDNF = Brain-derived neuronal factor

b-FGF = Basic fibroblast Growth Factor

BSA = Bovine Serum Albumin

C = solute concentration, M L^{-3}

CNS = Central Nervous System

DAPI = 4'-6-Diamidino-2-phenylindole

DMA = Dimethylamide

DMF =Dimethylformammide

ELISA = Enzyme-Linked ImmunoSorbent Assay

FC = Fluorocarbon

FITC =Fluorescein isothyocianate

HFMs = Hollow fibers microporous membranes

LSCM = Confocal Scanning microscope

GAP43 = Growth-associated protein-43

J_V = volume flux, M L^{-2} T^{-1}

Lp = membrane hydraulic permeability, T L^{-1}

P = hydrodynamic pressure, M L^{-1} T^{-2}

PAN = Polyacrilonitrile

PEG = Polyethylene glycol

PEEK-WC = Polyetheretherketone

PLL = Poly-L-lysine

PND = post natal days

PSCD = Polystyrene culture dishes

SEM = Scanning Electron Microscope

Chapter 6

Hollow fibers membrane for culture of hippocampal cells

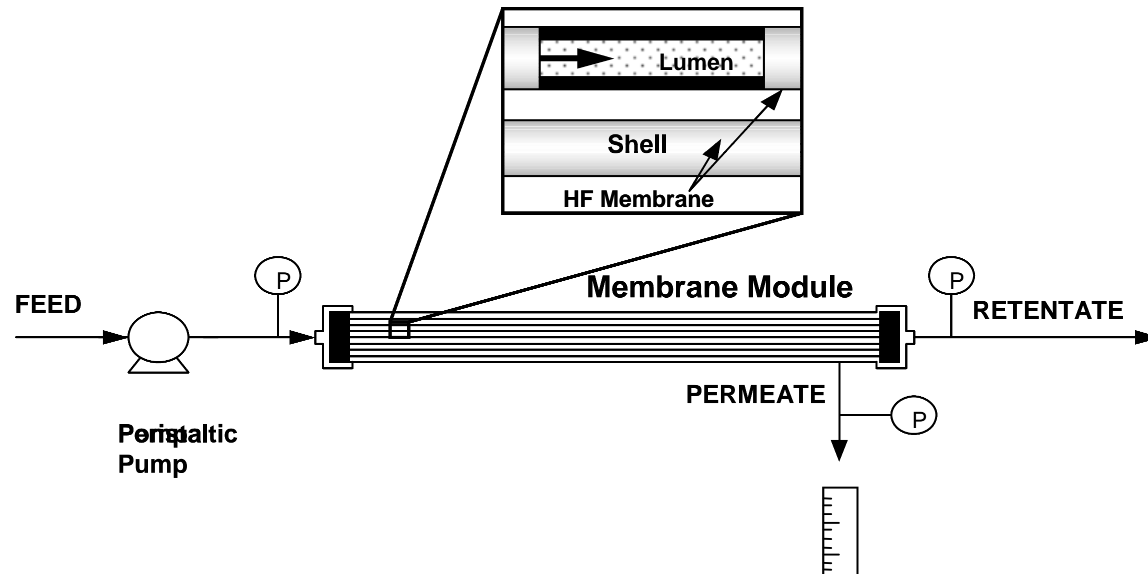


Figure 1 Schematic diagram of the experimental setup

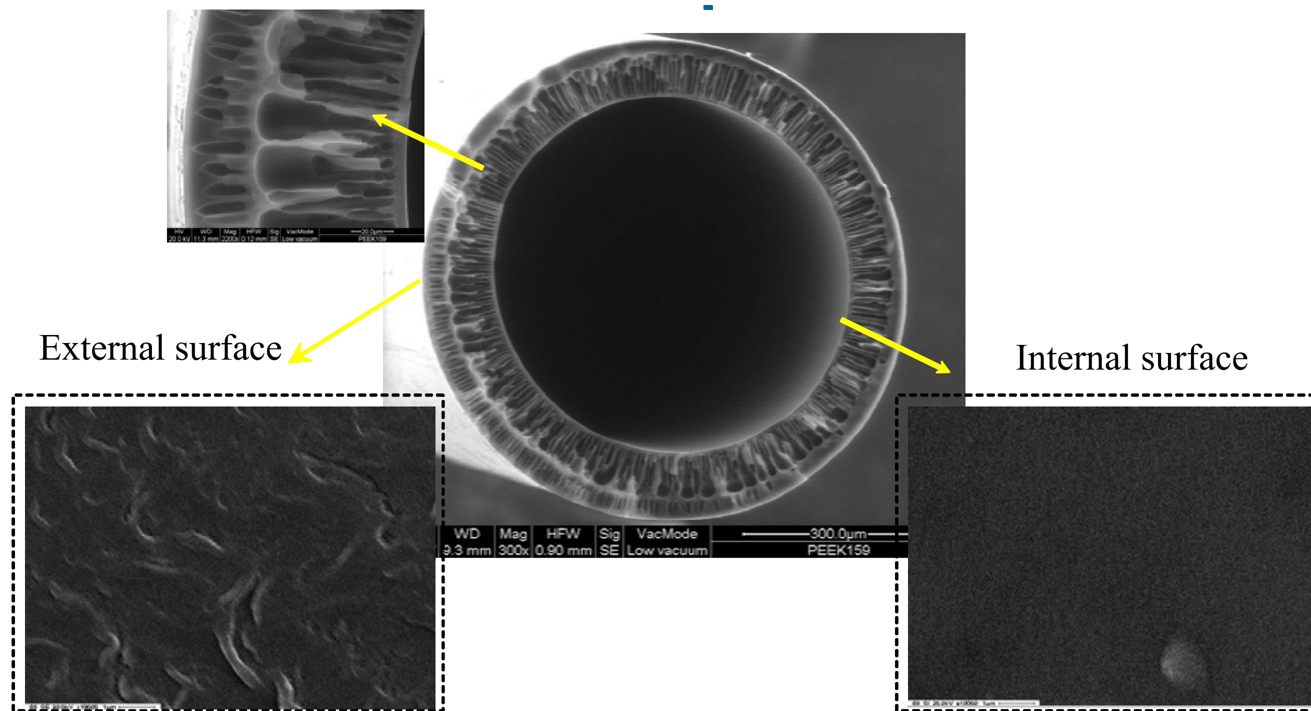
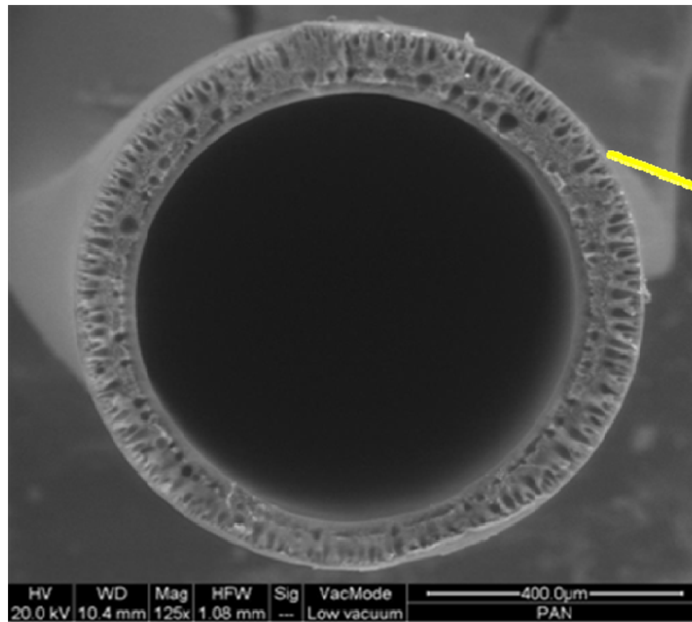


Figure 2 Representative scanning electron micrographs of cross-section, and external and internal surfaces, of PEEK-WC-HFMs.

Membrane Cross-section



External surface

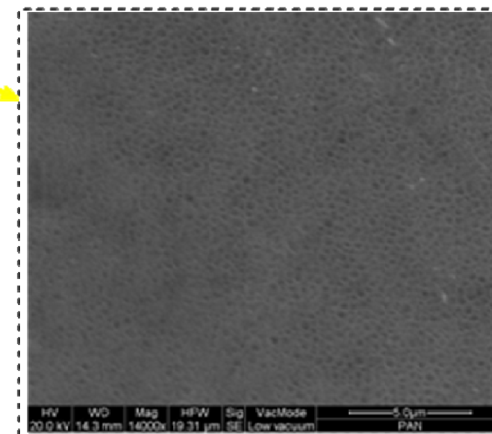


Figure 3 Representative Scanning electron micrographs of cross-section of PAN-HFMs.

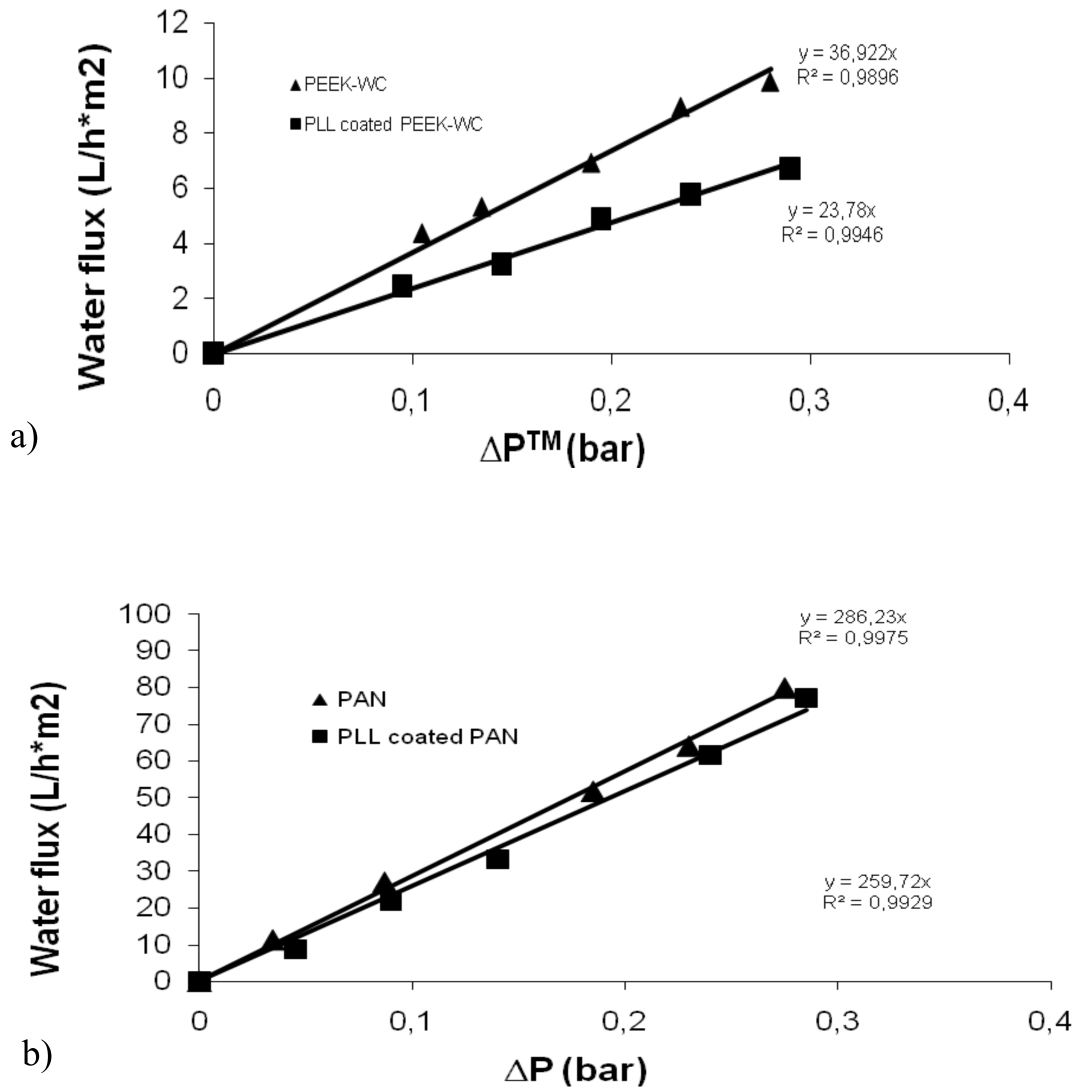


Figure 4 Hydraulic permeation measurements of a) PEEK-WC-HFMs before (▲) and after poly-L-lysine coating (■) and b) PAN-HFMs before (▲) and after poly-L-lysine coating (■) membranes. The experimental values were averaged on 10 measurements. The interpolation of experimental data is reported as solid line.

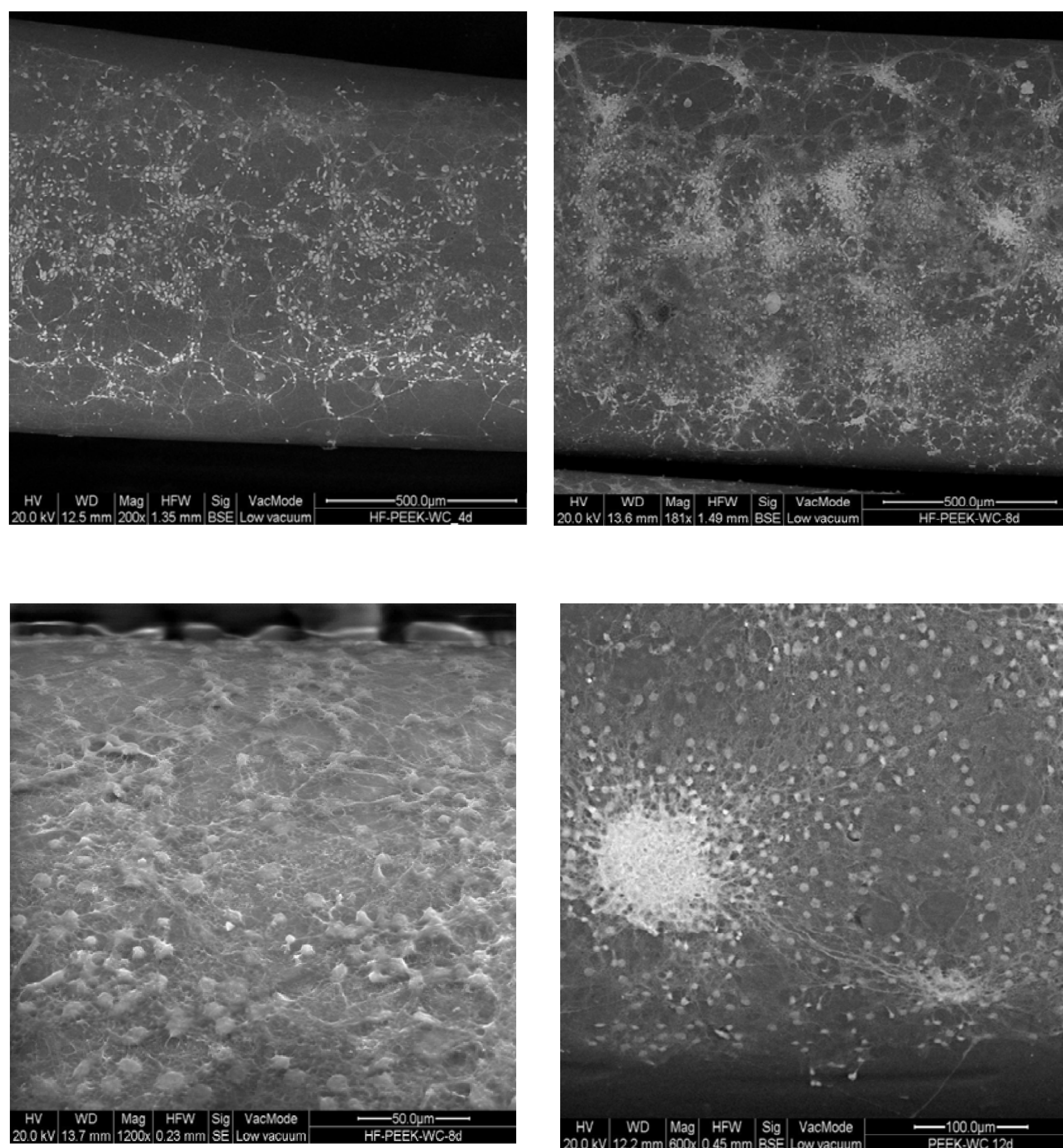


Figure 5 Micrographs of hippocampal neurons on PEEK-WC-HFMs after a) 4 days, b) 8 days c), and d) 12 days of culture. The arrows in a) indicate the axon (black) and the dendrites (white).

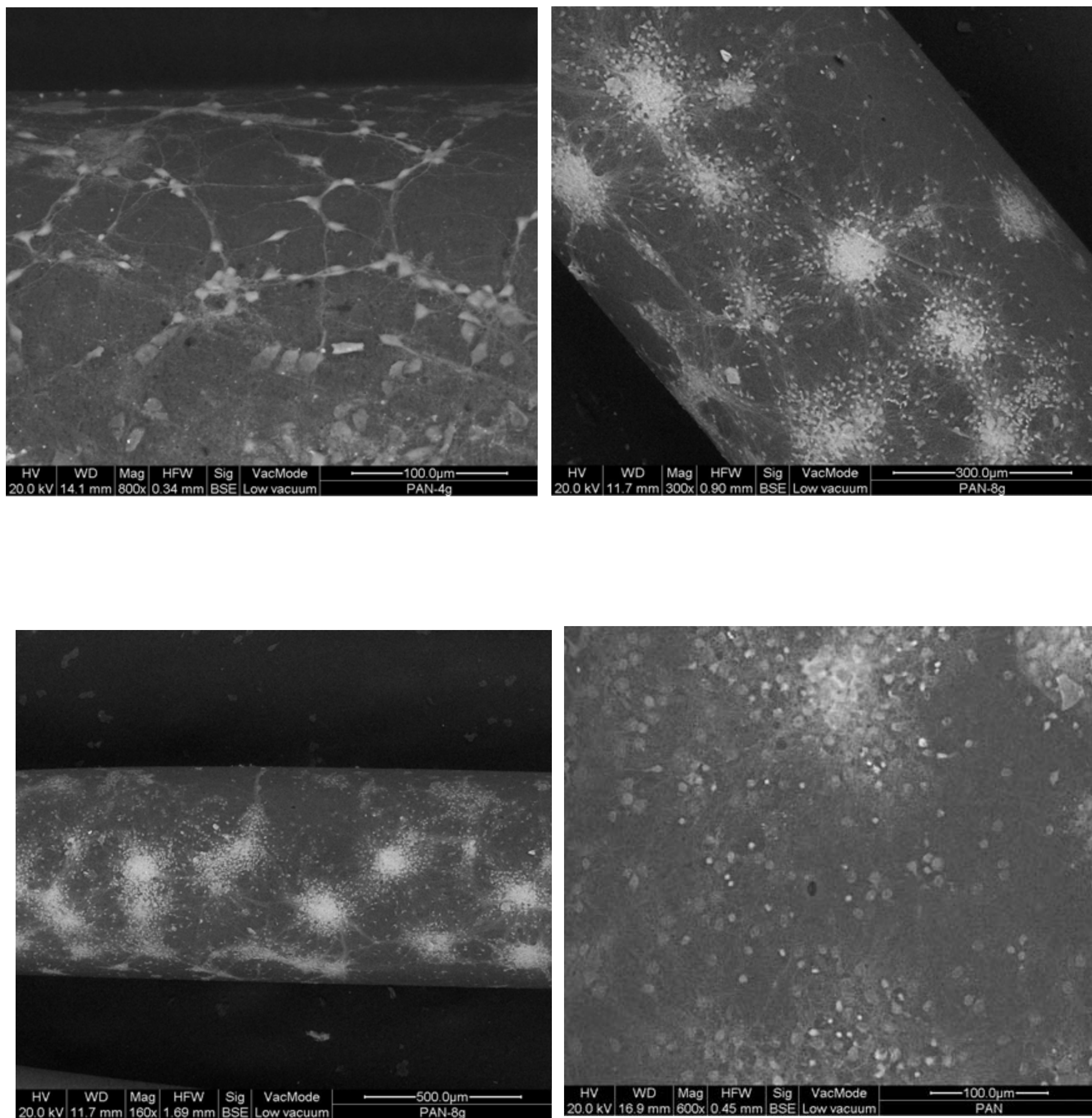


Figure 6 Micrographs of hippocampal neurons on PAN-HFMs membrane after a) 4 days, b) 8 days, c) 8 days, and d) 12 days of culture. The arrows in a) indicate the axon (black) and the dendrites (white).

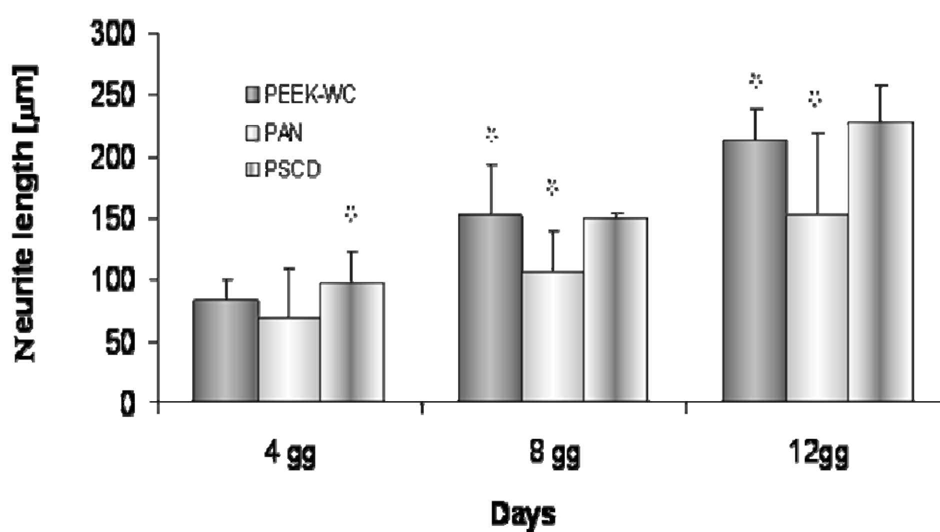
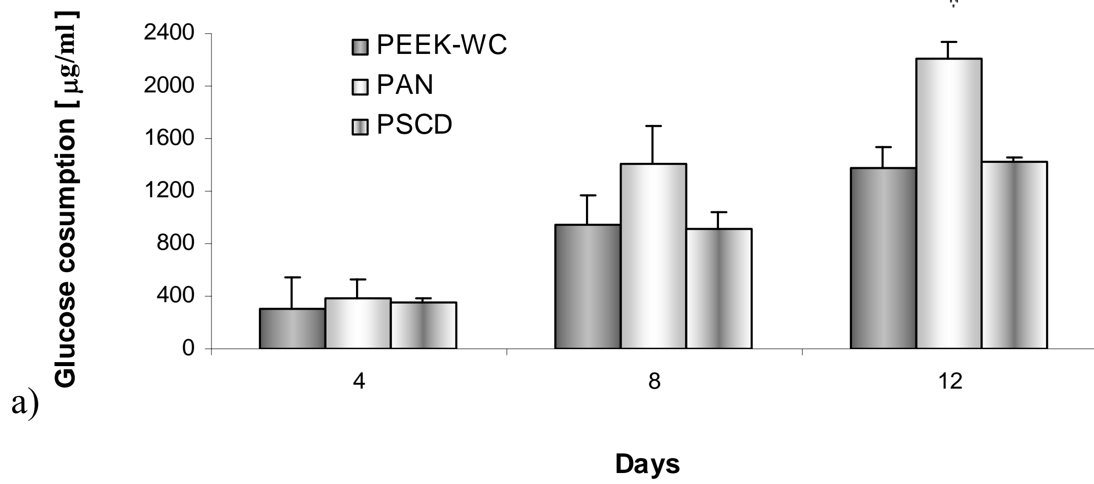
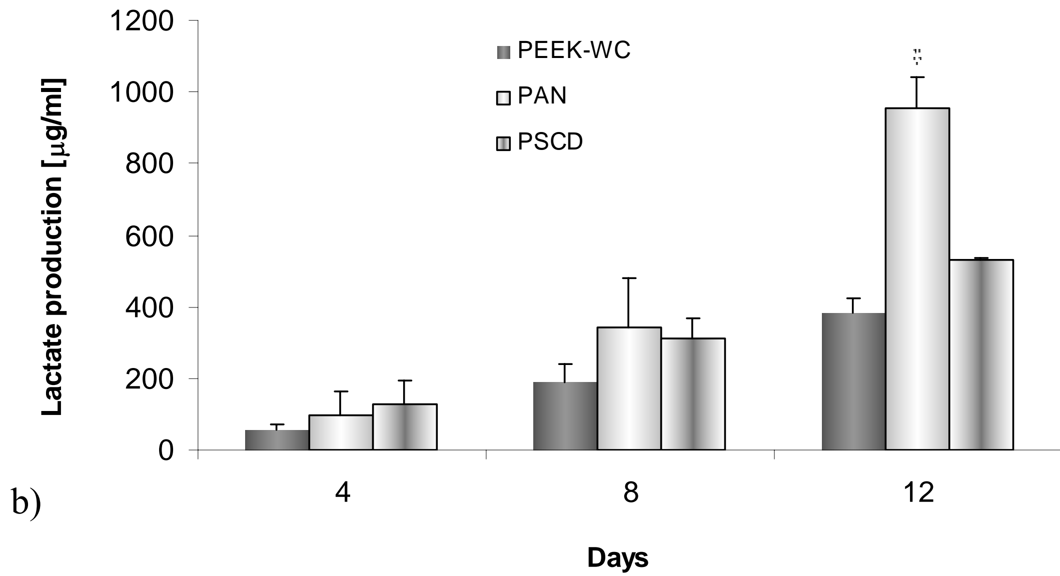


Figure 7 Axonal length of hippocampal neurons after 4, 8 and12 days of culture on the different surfaces. Data were expressed as µm ± s.e.m. and evaluated according to ANOVA followed by Bonferroni *t*-test. *$p<0.05$ versus PAN-HFMs.


Glucose cosumption [μg/ml]
2400
2000
1600
1200
800
400
0
PEEK-WC
PAN
PSCD
4
8
12
Days
a)


Lactate production [μg/ml]
1200
1000
800
600
400
200
0
PEEK-WC
PAN
PSCD
4
8
12
Days
b)

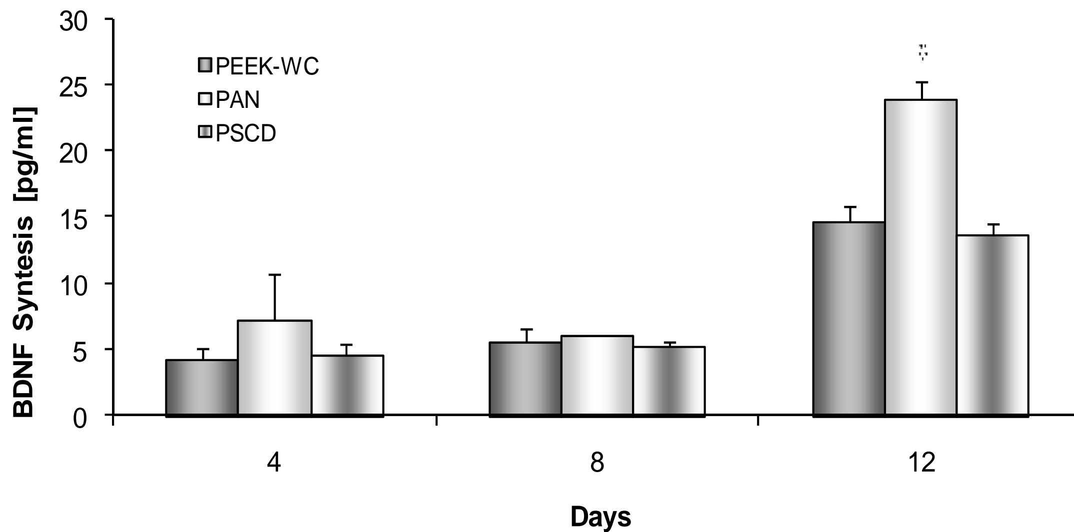


Figure 8 Metabolic activity of hippocampal neurons cultured on the different membranes.

a) Glucose consumption of hippocampal neurons on the different membranes on day 4, 8 day and day 12 of culture. The values expressed as µg/mL ± std.dev. are the mean of 6 experiments and evaluated according to ANOVA followed by Bonferroni *t*-test. *p<0.05 versus PEEK-WC-HFMs and PSCD.

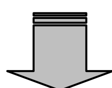
b) Lactate production of hippocampal neurons on the different membranes on day 4, 8 day and day 12 of culture. The values expressed as µg/mL ± std. dev. are the mean of 6 experiments and evaluated according to ANOVA followed by Bonferroni *t*-test. *p <0.05 versus PEEK-WC-HFMs and PSCD.

c) 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. and evaluated according to ANOVA followed by Bonferroni *t*-test. **p>0.05 versus PSCD; §p>0.05 versus PSCD; $^{+}$p>0.05 PEEK-WC-HFMs.

Morphological Membrane properties	PEEK-WC-HFMs	PAN-HFMs
Inner diameter	562.5 ± 9.6 μm;	683.2 ± 7.2 μm
Outside diameter	833.3 ± 5.8μm	891.7 ± 7.2μm
Wall thickness	135 ± 5μm	104.2 ± 7μm
Molecular cut-off	78000 Da	81000 Da

Table 1 Morphological properties of membrane

Number of laboratory animal	**Viable Cells N/ml**	**Dead Cells N /ml**	**Viable Cells (%)**	**Dead Cells (%)**
9	4.38 $x10^6$	1 $x10^5$	99,54	0,45
11	7.35 $x10^6$	3 $x10^5$	98,98	1,02
8	3.23 $x10^6$	4 $x10^5$	96,99	3,01
10	2.73 $x10^6$	0	100	0
13	6.27 $x10^6$	1 $x10^5$	98,43	1,57
11	8.02 $x10^6$	2 $x10^5$	97,57	2,43
6	7.40 $x10^6$	1 $x10^5$	98,67	1,33



98% Viability

Table 2 Representative table of cells viability

Chapter 7

Chapter 7

Chitosan biodegradable films for neuronal tissue regeneration

7.1 Abstract

Biomaterials that have been successfully employed in the manufacture of neuronal tissue include biodegradable materials as polyglycolic acid (PGA), poly-L-lactic acid (PLA), and poly(lactide-coglicolide) (PLGA). A potential advantage offered by biodegradable materials is the disappearance from the implant site once regeneration has been completed, obviating the foreign body response and the long–term possibility of infection-related complications.

In this work, i have proposed a new chitosan films for *in vitro* hippocampal cell growth. Chitosan (b-1,4-d-glucosamine) is a polysaccharide with excellent biological properties, has been widely used in biomedical fields.

The properties of chitosan films have been studied and the capacity to permitted the *in vitro* differentiation of hippocampal cells.

Neurons cells extract from hippocampus of *mesocricetus auratus* were cultured in chitosan (CS) membrane.

The effective biocompatibility of the material was demonstrated by analysis of morphological parameters of neuronal cells and evaluation of the metabolic activity of cells in culture.

Long-term hippocampal cell culture was obtained on CS, the cell growth and the metabolic functions are much similar to the natural substrate on CS surface. These demonstrate that cells on CS membrane growth in conditions culture similar to the tissue in vitro without proteins which promote cell adhesion and growth.

The results provide that this biodegradable chitosan films can been used for tissue engineering applications.

7.2 Introduction

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.

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Chitosan biodegradable films for neuronal tissue regeneration

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. For *in vivo* tissue regeneration, biodegradable materials are required. Biodegradable materials must be biocompatible and meet other important critic such as controlled degradation in response to biological conditions without release of toxic or unsafe degradation products.

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 [2]. 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 [1].

The Chitosan is an attractive polymer for its biocompatibility, water absorbent and because is naturally degraded by body enzymes.

In this study i report on the preparation of a chitosan membrane for promoting neuronal cell growth. To this purpose, a membrane made by blending of chitosan with polyethylene glycol was developed and characterized. Neurons used in this experiment were isolated from hippocampus.

The status of the cell growth on CS 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 (length) and viability. Metabolic behaviour of cells was evaluated by assessing glucose consumption, lactate production and secretion of brain-derived neuronal factor (BDNF). This neurotrophic factor is produced by hippocampal cells in the synapsis connections, during the development. BDNF supports the survival of existing neurons and encourages the growth and differentiation of new neurons and synapses [3]. 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 [4].
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.

7.3 Materials and Methods

7.3.1 Membranes preparation

Membranes were prepared by inverse phase techniques using the precipitation by evaporation of solvent [5]. Polymer solution is constituted by 4% (wt/wt) chitosan solutions and 1% of PEG 6000 in acetic acid at 2 % w/v concentration (wt/wt). Before chitosan was dissolved in 2 % acetic acid solution and then PEG was added to the chitosan solution. The mixing solution was cast on as an suitable support 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 [6].
Poly-L-lysine–coated Polystyrene culture dishes (PSCD) were used as a control. PSCD were coated 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 surface with a density of 40 $\mu g/cm^2$ [7]. 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..

7.3.2 Membrane characterization

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. The 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:

$$\text{surface water sorption (\%)} = \frac{\text{drop volume}_{(t=0)} - \text{drop volume}_{(t=i)}}{\text{drop volume}_{(t=0)}} * 100 \qquad [5]$$

7.3.3 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 [8]. 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 [9]. 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 [10, 11]. 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.

7.3.4 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 anti-βIII-tubulin followed by secondary antibody conjugated with FITC fluorochrome on samples previously fixed and permeabilized [4,12]. 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 anti-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 [9, 12].

7.3.5 Neuronal morphology CS 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 ± std.dev.).

7.3.6 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.*

7.3.7 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 *t*-test ($p < 0.05$).

7.4 Results

The morphology of the CS membrane was evaluated by observation of SEM images. CS membranes are characterized by the presence of microstructure over the surface and the absence of pores (Fig.1). Membranes have characteristics of transparency therefore permit the observation of the cells with time by light microscope.

Chapter 7

Chitosan biodegradable films for neuronal tissue regeneration

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. Chitosan membrane surface has a moderate wettability as demonstrated by time –related contact angle (Fig. 2a). At t=0 the value of contact was 55.8° ± 5.6°, that decrease at value of 42.2° ± 1.8° after 2.88 sec. The water sorption over the surface increased with time reaching-volume of 18% after 1,28 s (Fig.2b).
The viability of primary hippocampal neuronal cells isolated from 1, 2 days year's old *mesocricetus auratus* was 98.7%. The morphological changes that occur in the development of hippocampal neurons in culture on CS membranes are shown in Fig.3.
In fig. 3a, after 4h of culture, cells emerge to the CS membranes appeared with spherical shape and from several sites of circumpherence "minor processes" start to attached. At day 4 of culture the polarization of cells was evident, and the cell's processes differentiated axons and dendrites (See arrows in Fig. 3b). Neurites continued to grow at rapid rate, but the remaining minor processes undergo little net elongation.
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 (Fig. 3d). 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 membrane is suitable for the neuronal development.
Axons frequently course along the dendrites and, by light microscopy, such axons cannot be distinguished from the underlying 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.
The confocal images of the neurons, showed the localization of βIII-tubulin (green) in the neuronal network (Fig.6). 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 confirm this important result, the morphology of cells was analyzed, by measure of size of cellular body and neurites outgrowth (Fig.6). The soma area is smaller in CS respect to PSCD, demonstrating the absence of cell spreading (6a). Also neuronal network developed above the membrane surface at similar levels to PSCD and the cells exhibited a good neurite outgrowth although on the membrane cells seem shorter neurites than those developed PSCD (6b). Neurons on CS membranes exhibited a basal metabolic activity similar to the control substrates. Significant differences were observed than day 8 to day 16 for glucose consumption between CS membrane and PSCD (Fig. 7a). Cells on control substrates exhibited higher glucose consumption with respect to PSCD. The lactate production rate is similar on CS then PSCD throughout the culture period (7b). Differently during the first days of culture, the BDNF secretions is higher on CS membranes than PSCD, the production of BDNF decrease with time on this substrate (Fig. 7c). The evaluation of the metabolic activity demostrated that the membrane is able to support the cells functions throughout the entire culture period.

7.5 Discussion

This study demonstrates the successful adhesion and growth of primary neurons isolated from the hippocampus on CS membranes. Biodegradable membrane was prepared blending Chitosan with PEG. The PEG was added in order to improve the mechanical properties of the membrane. CS is only soluble in aqueous medium on the presence of a small amount of acid such as acetic acid and its mechanical properties are not optimal. PEG is a water soluble polymer that exhibits low toxicity and immunogenicity. Because of the good biological activities of CS and PEG , a combination of both polymers resulted in a beneficial effects on the biological and physio-chemical characteristics of the complex membrane.

CS Membrane was prepared with phase inverse technique and characterized with SEM for evaluation of membrane morphology. At SEM's analysis CS membranes appeared with dense and homogeneous structure, particularly interesting for hippocampal cells culture [1].

The transparency characteristics of this membrane that allow to observe cells by light microscope and monitor morphological changes (Fig.1).

Chitosan biodegradable films for neuronal tissue regeneration

The phisico-chemical characterization confirmed the hydrophilic nature of this membrane that makes it to be suitable for cells (Fig.2).

CS membrane bio-hybrid system promotes cell response and cell interactions without poly-L-lysine coating (Fig.3). Many biomaterials, are coated with different extracellular matrix proteins for promote the adhesion and modulate the growth, [13-14]. These results demonstrated that developed CS membrane is able to favour the cell differentiation in absence of permissive adhesion's protein.

The cyto-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 on CS membrane (Fig. 3). The density of axonal network increases with the time, and neurites become more elaborate and highly branched on the membrane.

In contrast with the reference substrate neurons tend to develop their extend processes into the membrane matrix [15]. This result involve on underestimation of neurite length extended the membrane (Fig.6b).

Neuronal cells on CS 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 and develop their extend processes into the membrane matrix. As a result, neurites are not completely visible over the membrane surface.

It is interesting to note that hippocampal cells can growth and differentiate, extending neurites, on biocompatible surfaces without coating of proteins such as PLL (Fig.4).

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 (Fig.5).

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. 7). 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 (Fig.7a,7b). 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 (7c) [16]. In the brain, it is active in the hippocampus, cortex, and basal forebrain areas vital to learning, memory, and higher thinking [9]. BDNF itself is important for long-term memory [17]. 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 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 demonstrated that cells on CS membrane have a maturation stage faster than hippocampal cells on PSCD, developed highly branched neurites and a more complex network.

7.6 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 highly branched neurites. 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. These results demonstrated that the CS membrane can be used to promote cell differentiation and cell neurite outgrowth. The advantage of this membrane is that it can be used without further modification with coating of ECM proteins. The membrane characteristics of biodegradability can be used to promote *in vivo* neuronal regeneration besides of using to the development neuronal tissue engineered construct *in vitro*.

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Chapter 7

Chitosan biodegradable films for neuronal tissue regeneration

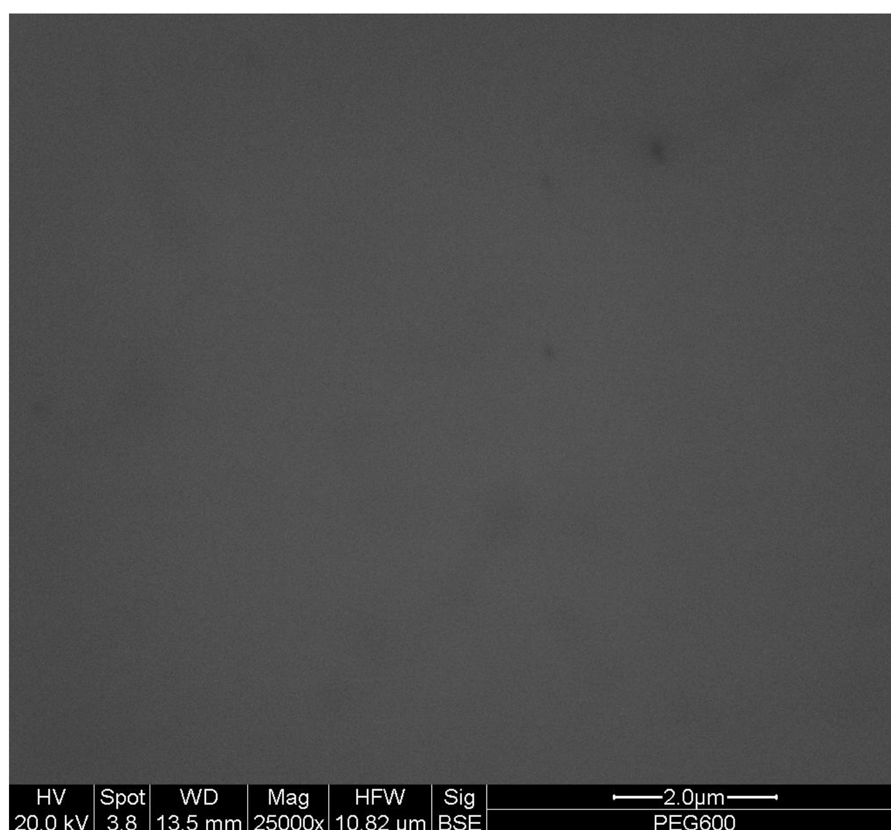


Figure 1 Scanning electron micrographs of CS surface membrane.

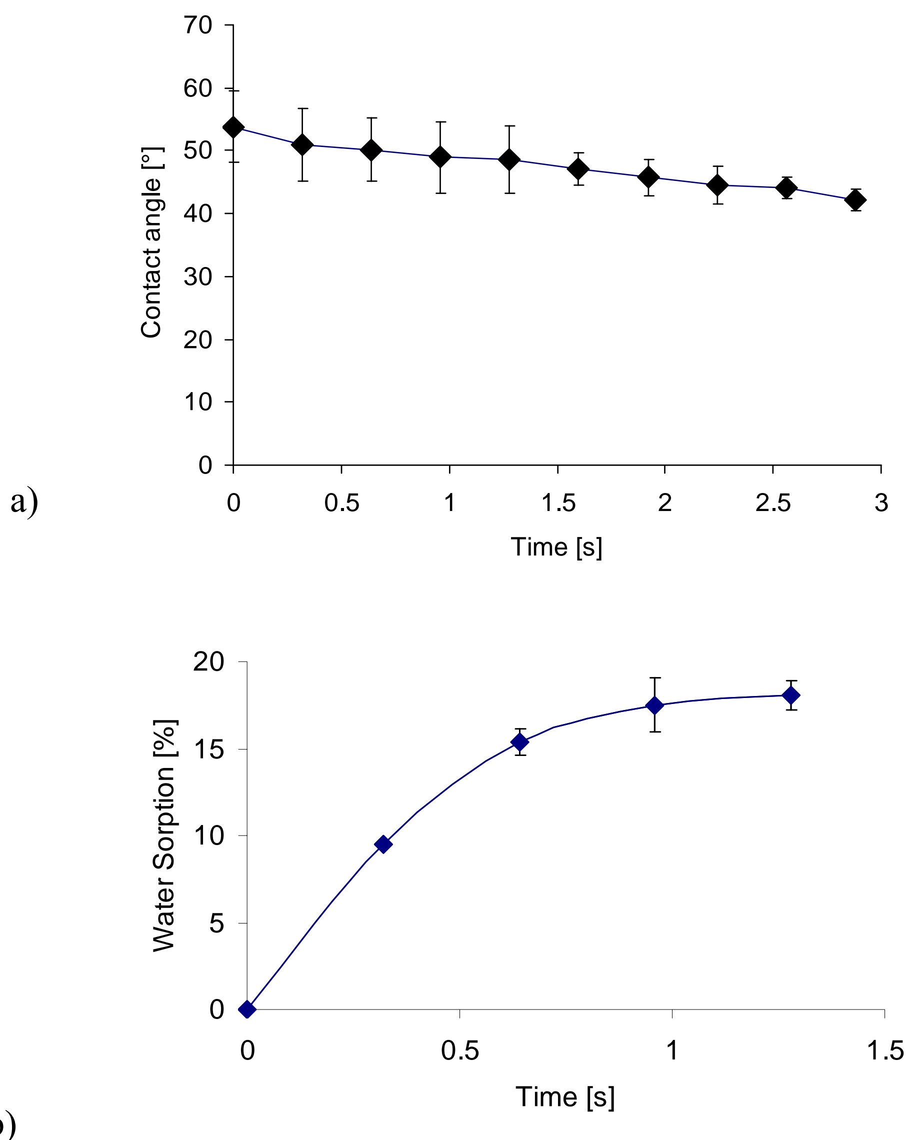


Figure 2 a) Time-related water contact angles measured on chitosan membrane surface. The reported values are the mean of 30 measurements of different droplets on different surface regions of each sample ± std. dev. b) Surface water sorption percentage of chitosan membrane. The reported values are the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

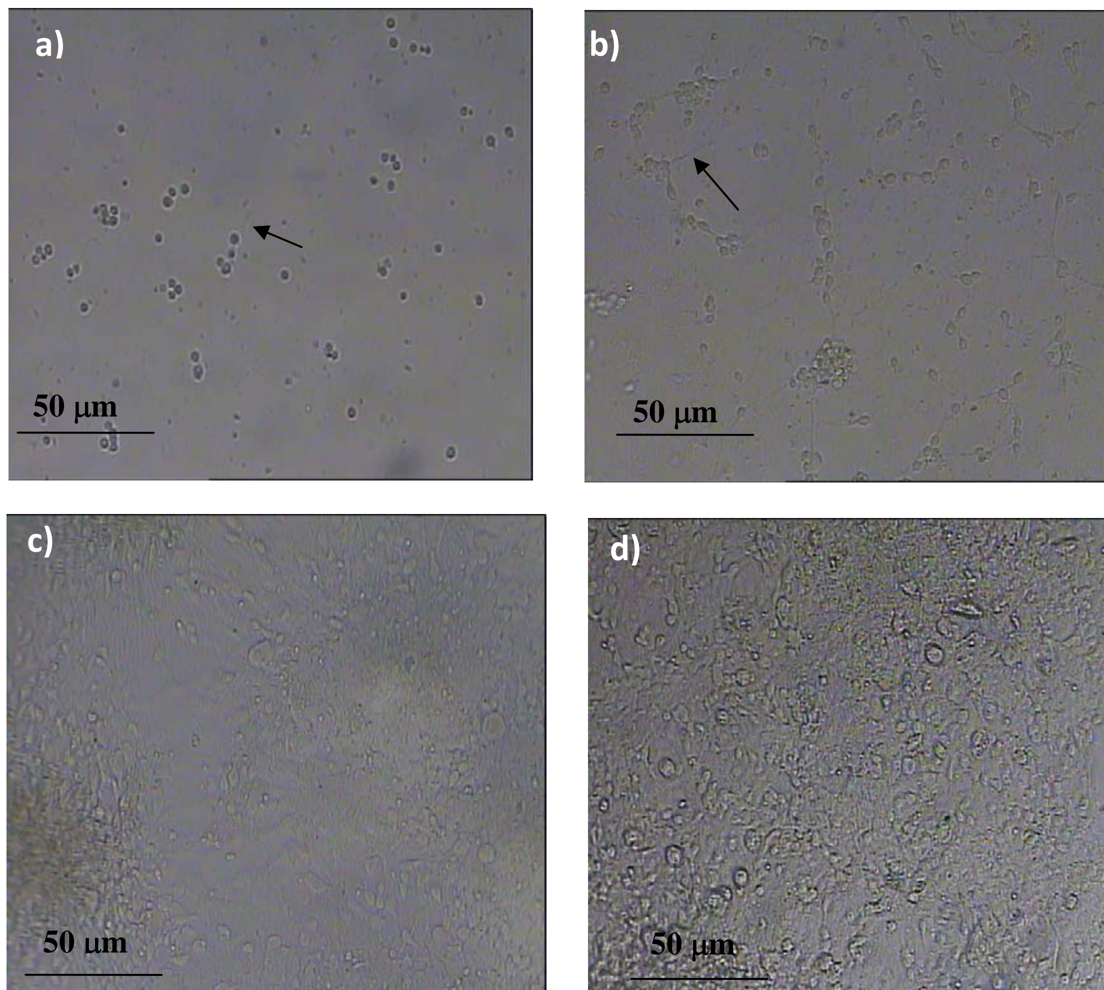


Figure 3 Micrographs of hippocampal neurons on CS membrane after a) 4 hrs, b) 4 days, c) 8 days, d) 16 days of culture. The arrows in a) indicate the emerging processes from the cell circumference; the arrows in a) and b) indicate the neurites.

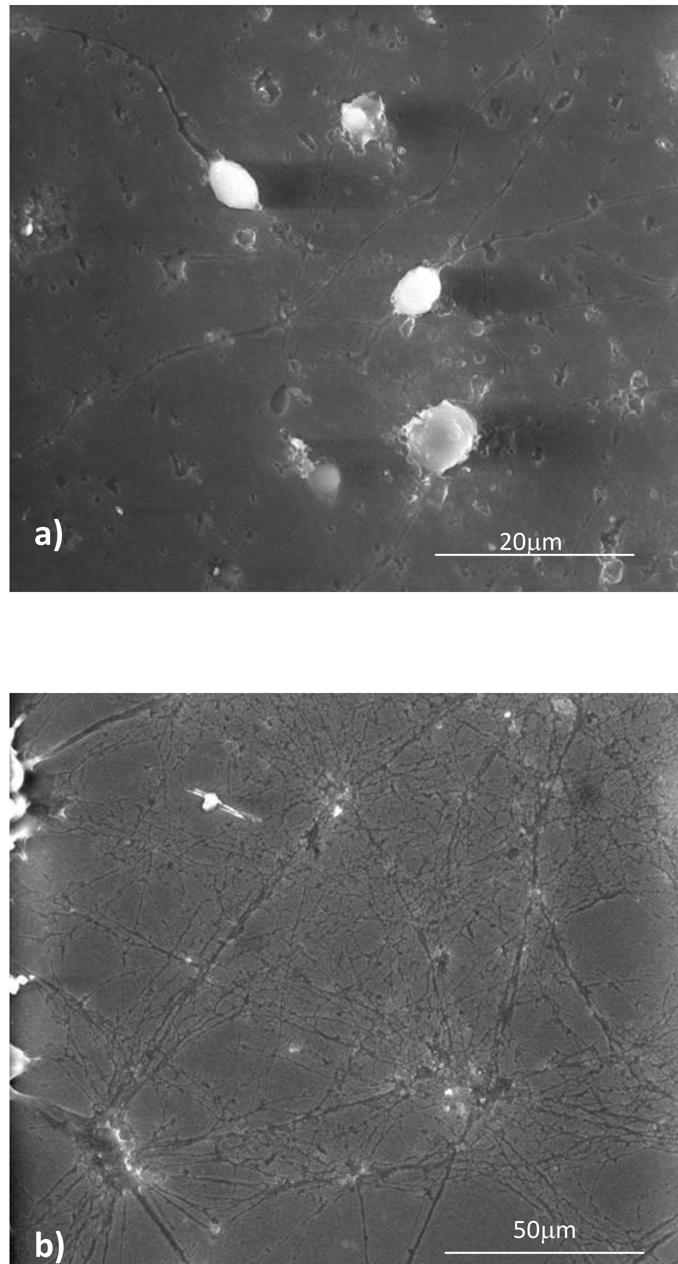


Figure 4 SEM's images of hippocampal neurons on: a) CS after 4 days of culture (4000X) and b) CS after 8 days of culture (1600X).

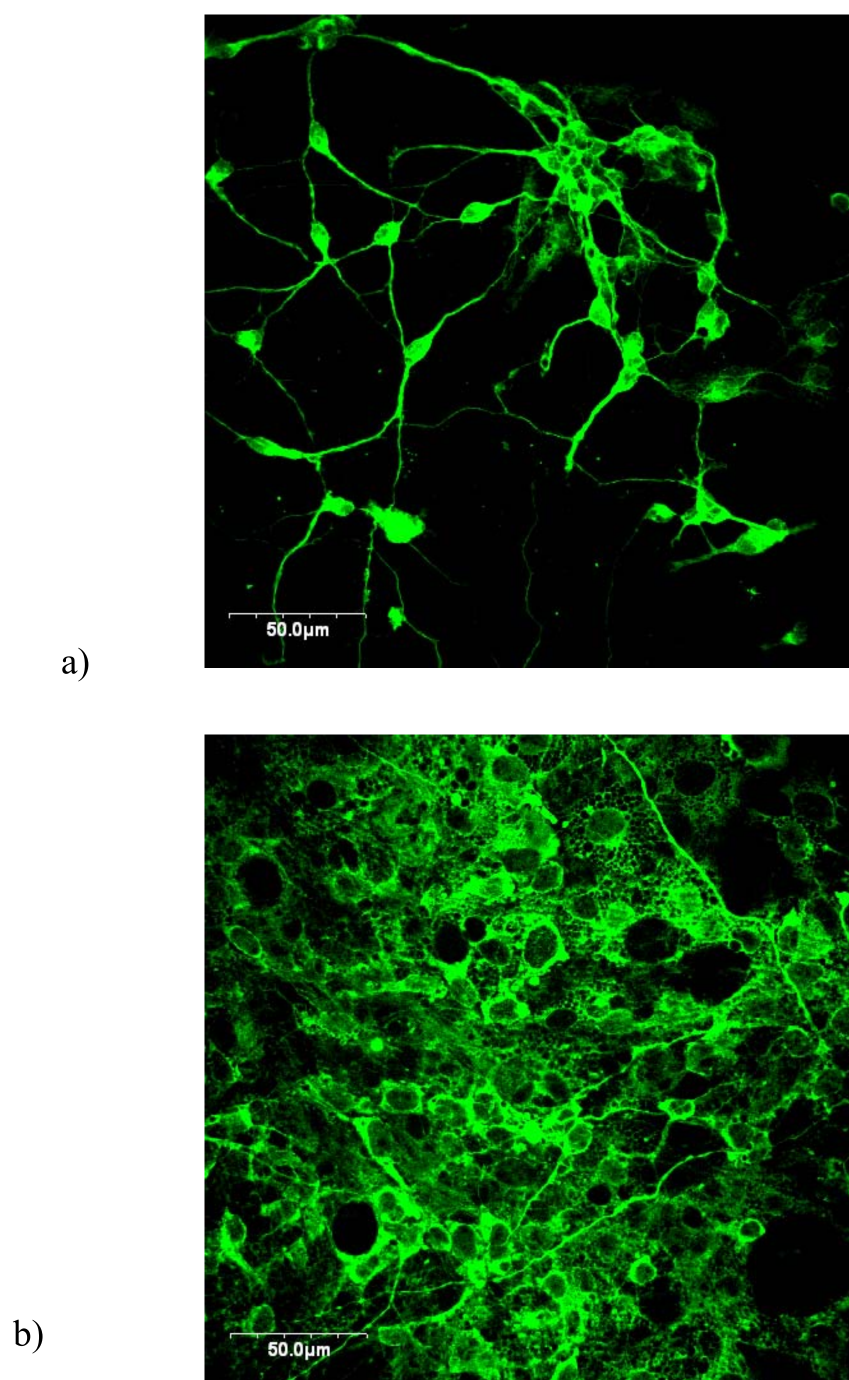


Figure 5 Confocal laser micrographs of hippocampal neurons traced for entire culture period and from representative images after 4 and 16 days of culture it was possible to evaluate the distribution of βIII-tubulin (green) on CS membranes.

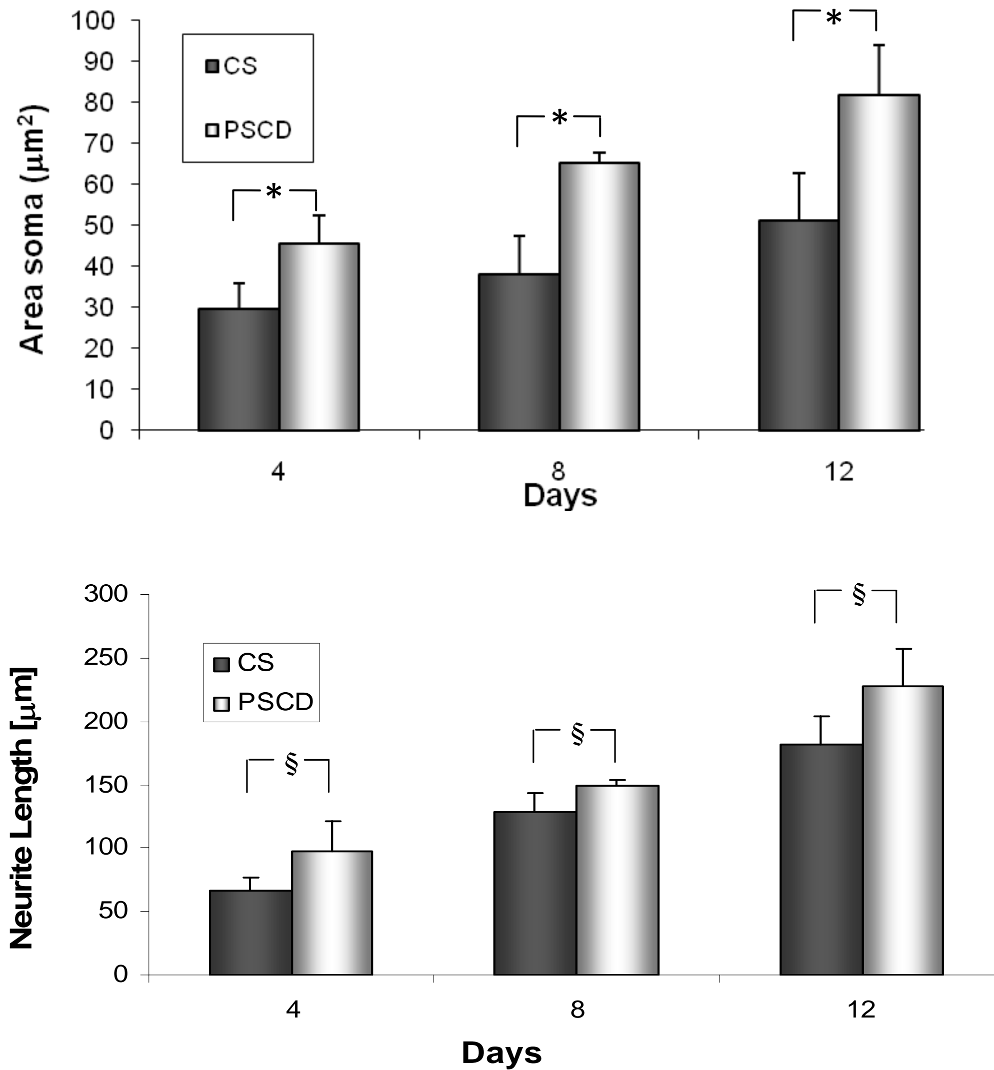
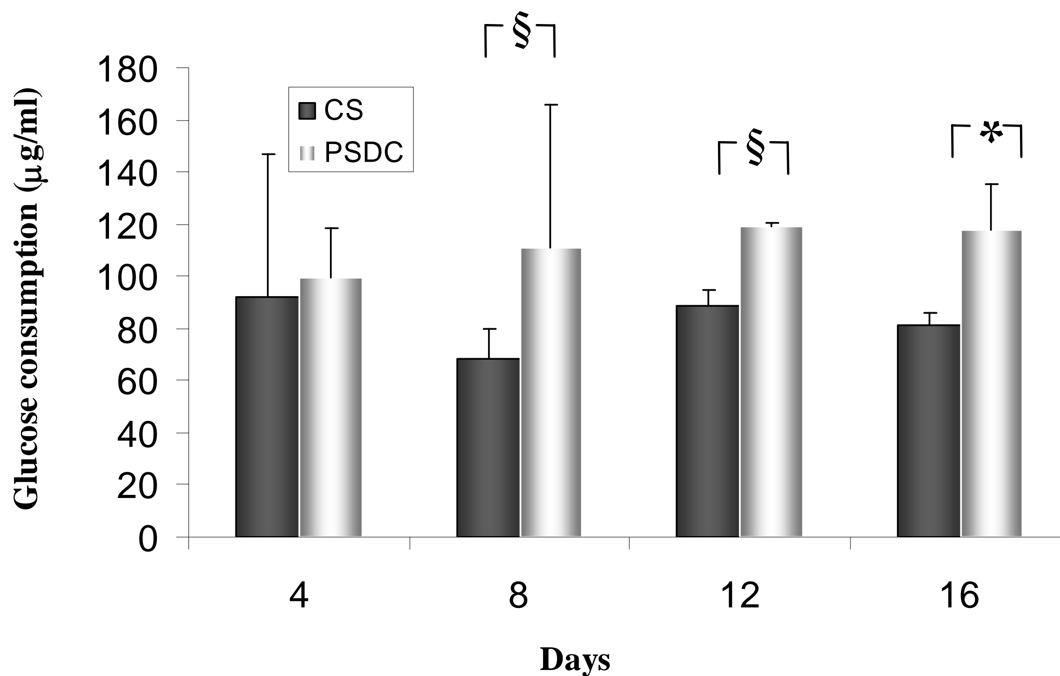


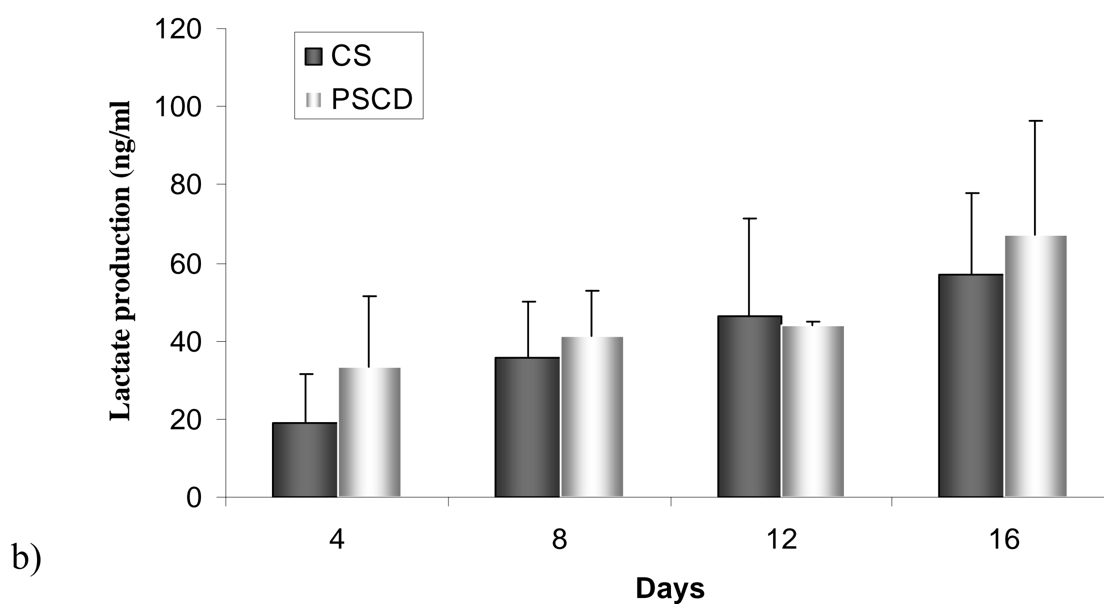
Figure 6 Analysis of morphological parameters of hippocampal cells on CS, CS-Ply and PSCD surfaces:

a) Area soma of hippocampal neurons after on day 4, 8, 12 of culture on CS membrane and PSCD. Data were expressed as μm ±std.dev. and evaluated according to *t* –test. *p<0.0001.

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



a)



b)

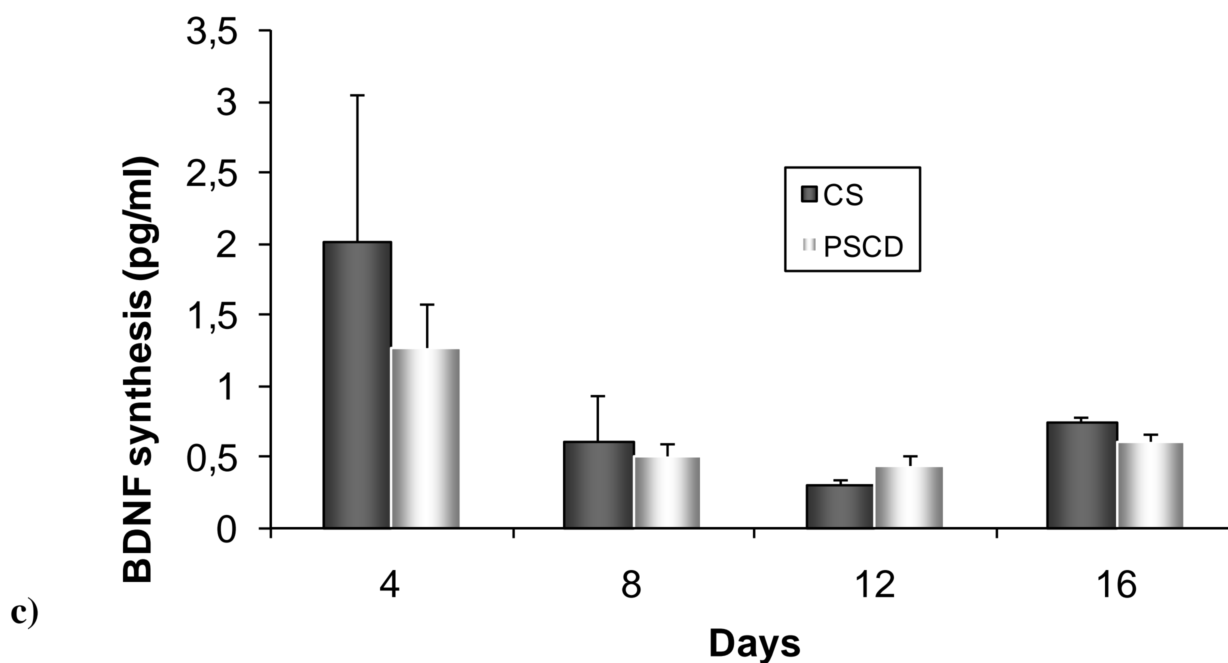


Figure 7 Metabolic activity of hippocampal neurons cultured on the different membranes.
a) Glucose consumption of hippocampal neurons on the different membranes on CS membrane and PSCD on day 4-8-12-16 of culture. The values expressed as μg/mL ±std.dev. are the mean of 6 experiments and evaluated according to *t*-test. * $p<0.05$; § $p<0.001$.
b) Lactate production of hippocampal neurons on CS membrane and PSCD on day 4, 8 , 12, 16 of culture. The values expressed as μg/mL ±std. dev. are the mean of 6 experiments.
c) BDNF secretion of hippocampal neurons on CS membrane and PSCD on day 4-8-12-16 of culture. Data were expressed as pg/mL ± std. dev. are the mean of 6 experiments

Chapter 8

Chapter 8

Conclusions and Future Research

8.1 Conclusions

Membrane bio-hybrid systems constituted of neuronal cells isolated from hippocampus and semipermeable membranes in flat and hollow fiber configurations were developed. Membranes were used to favour the adhesion and compartimentalization of cells and the regeneration of neuronal network.

A prerequisite for the development of the neuronal membrane bio-hybrid system is the optimization of cell isolation and culture. A protocol of isolation and culture of hippocampal neurons has been optimized by using a hibernating rodent, the hamster *Mesocricetus auratus* as our animal model. This rodent is a useful model to provide new insights into neurodegenerative disorders such as ischemia, which occurs during the arousal bout in hibernators. After optimization of isolation procedure $98 \pm 2\%$ viable cells were obtained. These results of the optimized protocol demonstrated that the plating density of 256000 cell/cm^2 was optimal for the survival and differentiation, at least of this type of neurons.

The use of membranes in tissue engineering and medical fields requires materials with high biocompatibility and bio-stability properties. Novel membranes were prepared from a polymeric blend of modified polyetheretherketone (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 techniques. These membranes offer the advantage of combining the properties of both polymers (biocompatibility, thermal and mechanical resistance, elasticity) with those of membranes such as permeability, selectivity and well-defined geometry of pores. Hippocampal pyramidal cells were plated on this membrane; the result suggest that morphology and physiological properties of hippocampal cells, which appear to be very sensitive to an inadequate culture media, have been not altered on membranes and this relationship strongly supports the value of such a bio-hybrid substrate for long-term survival of neuronal cells and this means a more favorable condition to handle their neuro-

transcriptional events within time. Morphological and physico-chemical properties of the membrane influence the viability and differentiation of neuronal cells in *in vitro* membrane constructs. For this reason membranes with different morphological surface properties in terms of pore size, porosity and roughness were proposed as substrates for cell adhesion in the membrane biohybrid system.

Commercial microporous membranes such as Polyester (PE) and Polyethersulfone (PES) and a dense membrane permeable to oxygen, carbon dioxide and aqueous vapour such as Fluorocarbon (FC) were used together with membranes prepared from modified polyetheretherketone (PEEK-WC).

Neuronal cells respond to the different membrane surface by changing their morphology and neurite outgrowth. Neurons on the FC (Ra= 6nm) and on PES (Ra=49.38nm) membranes developed axons and highly branched dendrites and formed a complex network of neurite as well as on PSCD (control). While on the PE membrane surface (Ra= 87.2nm), cells adhered forming processes over the membrane surface that established intercellular contacts. Conversely, on the PEEK-WC membranes (Ra=200 nm), cells developed short neurites with the tendency to grow into the pores of the membrane surface. The specific metabolic functions show that the smoother membranes influenced functional maturation of hippocampal cells. Our results obtained with hippocampal cells showed an improved neuronal growth on smooth surfaces such as FC and PES membranes. The biohybrid system constituted by hippocampal neurons and FC membrane that gave the best performance was used to elucidate the neurogenic role of $GABA_A$ α_2 and α_5 subunits on the different hippocampal neuronal elements of the golden hamster. To this purpose a highly specific α_2 (flunitrazepam) agonist and an α_5 (RY-080) selective inverse agonist were used on hamster hippocampal neuronal. The $\alpha_{2,5}$ GABAergic and Gluergic subunits are responsible for the successful elongation processes of neuronal elements in hamster HIP cells. The results showed that in particular during the first days of culture activity, GABAergic ($\alpha_{2,5}$) and Gluergic (NR1/GluR2) systems were very crucial element for regulating axonal sprouting, dendritic protrusion motility and above all the early activation of silent synapses. The highlighting of the neuronal developmental processes guided by these two major inhibitory and excitatory circuits proposed the novel molecular and synaptic plasticity

mechanisms influencing hippocampal genomic neuronal programs of the hamster that may prove to be critical above all during some stages of hibernation.

Membranes in hollow fiber configurations are particularly advantageous in the application of neuronal regeneration owing their tubular architecture that gives a topographical guidance to the regenerating axons. Hollow fiber membranes offer to the cells: i) a wide surface area for the adhesion and for the mass exchange in a small volume, ii) the compartmentalization in a well defined microstructure, iii) perfusion without shear stress. The molecular weight can be adjusted to allow diffusion of nutrient, waste products, and the external humoral factors that are beneficial to regeneration across the membrane tissue interface, but exclude the transport of large immunogeneic molecules.

Hollow fiber membranes can be used to realize *in vitro* a tissue engineered construct in which neuronal cells are continuously perfused with nutrients and metabolites.

Hollow fibers microporous membranes (HFMs) were prepared for supporting the growth of hippocampal neuronal cells. The polymeric substrates were evaluated for their ability to control hippocampal neuronal cell adhesion and direct axon regeneration. Neuronal cells adhered outside the HF membrane surface and develop a typical well-definite shape of the hippocampal neurons, with a primary apical axon and many branched dentrites. With subsequent development, the density of the axonal network increased, dentritic arbors became more elaborate and highly branched, synaptic contacts developed in a large number propagating the neuronal network. The metabolic activity of neurons on membranes demonstrated that cells adhered on the membranes were functionally active for 12 days of culture. Results of this study encourage the development of a membrane bioreactor for hippocampal neurons growth, which is able to regenerate neural tissue in a well-controlled microenvironment and tested the effects of develop neuronal network in presence of physical and chemical stimuli too.

A biodegradable material was proposed for the capacity to permit the *in vivo* regeneration and differentiation of hippocampal cells. Chitosan (b-1,4-d-glucosamine) is a polysaccharide with excellent biological properties, has been widely used in biomedical fields. CS membranes that were developed were transparent and characterized by the presence of microstructure over the surface and the absence of pores.

Long-term hippocampal cell culture was obtained on this membrane and the effectively biocompatibility of the materials was demonstrated by analysis of morphological parameters of neuronal cells and evaluation of metabolic activity of cells in culture. Cells increased 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 was highly dependent on cell interactions with the substrate; confocal and SEM's micrographs demonstrated that CS membrane was suitable for the neuronal development. These results demonstrated that the CS membrane can be used to promote cell differentiation and cell neurite outgrowth. The advantage of this membrane is that it can be used without further modification with coating of ECM proteins and thanks to its biodegradability properties can be used to promote *in vivo* neuronal regeneration besides of using to the development neuronal tissue engineered construct *in vitro*.

8.2 Future Works

Neuronal membrane biohybrid system can be used for the *in vitro* study of neurodegenerative diseases like Parkinson's, Alzheimer's or Tay Sach's disease and epilepsy and the development of appropriate bio-molecules for therapeutic purposes.

The general medical doctrine has been that damage to central nervous system, including the brain and spinal cord, is irreversible. A major impediment to healing is the inability of adult neurons to proliferate in *vivo* and to be cultivated *in vitro*. The emergence of neuronal stem cell biology in the adult mammal has broken this barrier. The combination of neuronal stem cell biology with the applied principles of tissue engineering will revolutionize the medical approach to the treatment of damaged central nervous system tissue, including injuries or disease of both the brain and the spinal cord. Neuronal stem cells qualify an ideal cell for use in repair strategies of the central nervous system, including the spinal cord. Many of the characteristics that these unique cells possess make them ideal candidates for creation of functional central nervous system constructs. In the adult mammal these stem cells lay dormant in a quiescent state, but have the capacity to proliferate, producing mature cells with the properties of the damaged or injured tissue.

Realizing in vitro culture system of these cells in combination with biodegradable membrane can be the next step in a powerful reconstructive context regarding to neuronal replacement in damage or disease of central and peripheral nervous system.