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PAN hollow fiber membranes elicit functional hippocampal neuronal network

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Abstract This study focuses on the development of an advanced in vitro biohybrid culture model system based on the use of hollow fibre membranes (HFMs) and hippocampal neurons in order to promote the formation of a high density neuronal network. Polyacrylonitrile (PAN) and modified polyetheretherketone (PEEK-WC) membranes were prepared in hollow fibre configuration. The morphological and metabolic behaviour of hippocampal neurons cultured on PAN HF membranes were compared with those cultured on PEEK-WC HF. The differences of cell behaviour between HFMs were evidenced by the morphometric analysis in terms of axon length and also by the investigation of metabolic activity in terms of neurotrophin secretion. These findings suggested that PAN HFMs induced the in vitro reconstruction of very highly functional and complex neuronal networks. Thus, these biomaterials could potentially be used for the in vitro realization of a functional hippocampal tissue analogue for the study of neurobiological functions and/or neurodegenerative diseases.

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1 Introduction

Different tissue-engineering strategies have been developed in order to repair damaged axonal pathways in both, the peripheral nervous system (PNS) and the central nervous system (CNS). A promising strategy is represented by the possibility of creating a growth-permissive artificial substrate that acts as a guidance channel leading the spontaneous axon sprouting [1, 2]. To accomplish this task the artificial substrate must exhibit a specific micro-architecture able to stimulate nerve regeneration, therefore supporting axonal re-growth and affecting cellular orientation and differentiation in a positive way. Several works in the literature show evidence that different bridging strategies are useful to study neuronal behaviour for application in treatment of spinal cord injury and neurodegenerative diseases of CNS [1–4].

Recent trends for the investigation of neurobiological mechanisms in vitro have focused in the development of substrates that can control specific arrangements of neuronal axon growth and synaptic connectivity in neuronal networks. To improve the ability for regeneration of functional neuronal network and for the in vitro study of neuronal survival it is important to find a good biomaterial which is able to supply the conditions for normal neuronal differentiation and outgrowth [5]. Among the different biomaterials used for neurobiotechnology approaches, polymeric semipermeable membranes, both in flat and hollow fibre configuration, represent promising biomaterials for the reconstruction of a neuronal like tissue [6–8]. Recently, we demonstrated that membranes with high selective properties are suitable for the realization of well-developed neuronal network resembling

in vivo hippocampal neuronal properties [9–11]. In particular, semipermeable hollow fiber membranes (HFMs) can be used as guidance channels in promoting in vitro and in vivo axonal regeneration and creation of three-dimensional neuronal network [8, 12–14]. HFMs with selective properties provide a highly controlled microenvironment for the development of in vitro platform to be used in neuroscience and regenerative medicine.

In order to gain new insights on this topic, here we report about the development of an advanced in vitro neuronal model system based on the use of HFMs. We explored the ability of new HFMs of modified Polyetheretherketone (PEEK-WC) and Polyacrylonitrile (PAN) to promote the formation of a high density neuronal cell network directing axonal outgrowth. While the surface properties of the membranes are important in interactions with cells, the permeability characteristics control the cellular microenvironment by governing the mass transfer between cell compartment and external milieu. In order to tailor the neuronal response HF membranes prepared by the dry-wet spinning technique were modified by coated with poly-L-lysine (PLL) that is the substrate that allows the in vitro growth of neuronal cells [15].

The performance of the developed membranes in favouring and enhancing the reconstruction of a highly branched neuronal network were assessed by using primary hippocampal neurons. These cells are well known for their plasticity and regeneration properties [16] and are the best-characterised model for investigating polarization that occurs spontaneously during the first days of culture [17–19].

The creation of a functional neuronal network can be utilized to reproduce in vitro the same structural features that cells exhibit in vivo, displaying long neuritis processes outgrowth with numerous branches reaching a well defined shape typical of the hippocampal neurons.

Therefore, the determination of structural and cell features has been carried out through the evaluation of axon sprouting and neurite growth on PAN HF and PEEK-WC HF membranes up to 12 days of culture.

The specific neuronal functions of the membrane engineered system were evaluated by tracing the secretion of the brain derived neurotrophic factor (BDNF) which is an important indicator of the survival and differentiation of specific neuronal populations.

2 Materials and methods

2.1 HFM preparation

HFMs were prepared according to the well-known dry–wet spinning method by using the 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 PAN. PEEK-WC is a chemically stable polymer with excellent thermal and mechanical resistance [20].

Polymer solutions with 18% wt/wt of PEEK-WC in Dimethylacetamide (DMA) were prepared under continuous mechanical stirring at room temperature as previously described in [21]. HFMs were prepared by extruding the polymer solution through a spinneret in which dimensions (OD, ID, needle) were 800, 400 and 200 μm, respectively. A 50/50 v/v mixture of DMA and pure water was used as the bore fluid and tap water as the external coagulant. Polymer solution and bore fluid flow rates were 3.0 and 3.5 g/min, respectively. The air gap was 35 cm.

For the preparation of PAN HF membranes, a copolymer containing 92 and 8% wt/wt of acrylonytrile and vinyl acetate, respectively with a viscosity average molecular weight (M_V) of 40,000 Da, (Montefibre SpA, Italy) was used. Porous membranes were prepared by using PVP (K17 by BASF) as the pore forming additive and DMF as the solvent. The weight ratio was 15/15/70 for PAN, PVP and DMF, respectively. Polymer solutions were prepared by dispersing the PAN powder in DMF in a glass flask at room temperature to prevent the formation of large clots and afterwards PVP was added. The solution was then heated at 70°C under mechanical stirring until a homogeneous solution was obtained. The solution was then loaded onto a thermostated vessel and kept at 70°C. The spinning setup was the same for that used for PEEK-WC HF membranes. Polymer solution and bore fluid flow rates were 2.2 and 2.7 g/min, respectively. For the bore fluid, a 60/40 v/v mixture of DMF and pure water was used and the air gap was fixed at 60 cm. The membranes were modified by coating with PLL (MW 30,000–70,000), 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 μg/cm^2. The membranes were coated with poly-L-lysine in PBS and incubated for 3 h and then the excess of PLL solution was removed and dried.

2.2 Membrane characterization

The wettability of the native and modified membranes was characterized by means of water dynamic contact angle (DCA) measurements at room temperature with a CAM 200 contact angle meter (KSV Instruments Ltd., Helsinki, Finland). DCA measurements were performed under standard conditions, taking into account various parameters (e.g. temperature, cleanliness of sample, drop volume). The instrument supported by video camera and software permitted us to obtain 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.

The permeability properties of HFMs were characterized by pure water flux measurements in the absence of solutes and at different trans-membrane pressures (ΔP^{TM}). For each membrane, the hydraulic permeance L_p was evaluated before and after the modification process with PLL by applying the following equation [22]:

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

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

2.3 Cell isolation and culture

The hippocampus of both hemispheres was dissected from the brain of postnatal days 1–3 (PND1-3) hamsters (*Mesocricetus auratus*) (in accordance with the institutional and national guide for the care and use of laboratory animals), 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. Ten minutes after digestion, the tubes containing the tissue were 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), 5 ng/ml basic fibroblast growth factor (b-FGF; Sigma) was added to the remaining pellet. Samples were gently triturated mechanically 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 [7]. The samples were centrifuged at 1,300 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, 5 ng/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 [15, 19]. The viability of the cells after isolation was assessed by trypan blue test, which resulted to be 97 ± 2%. Cells were seeded on the HF membrane surfaces at 2.5×10^5 cell/cm^2 density. Controls without cells were prepared for each type 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 during each feeding interval.

2.4 Sample preparation for SEM

The morphological behavior of the neurons at 4, 8 and 12 days in vitro (DIV4, DIV8 and DIV12) on the different membranes was investigated by scanning electron microscope (SEM) (ESEM FEG QUANTA 200, FEI Company, Oregon, USA). Samples of neurons grown on PAN HF and PEEK-WC HF membranes were prepared for SEM 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 for the different membrane surfaces were obtained at DIV 4, DIV 8 and DIV 12.

2.5 Metabolic assays

The metabolic activity of neuronal cells was evaluated by assessing the neuronal BDNF levels in the culture medium from six different isolations and cultures, previously collected and stored in tubes at −20°C until assays. To assay BDNF secretion, a sensitive BDNF ELISAs immunoassay (Promega Corporation WI, USA) was carried out on samples collected at DIV 4, DIV8 and DIV12 according to the manufacturer protocol. The statistical significance of all experimental results was established by using ANOVA test followed by Bonferroni *t*-test ($P < 0.05$).

2.6 Morphometric measurements

Morphometric analyses in terms of axonal lengths of neurons cultured on the HFMs were performed after the immunostaining of neuronal axon marker, 43 kDa growth-associated protein (GAP-43) using a protocol as described in previous paper [9].

Representative images by laser confocal scanning microscopy (LCSM, Fluoview FV300, Olympus, Milan, Italy) displaying the distribution of GAP 43 in hippocampal cell growth on the different HFM surfaces were utilized at DIV8 and DIV12, periods in which the different neuronal elements and synaptic complexes respectively are fully formed. In particular, the axonal lengths were measured on the neurons stained with the axon marker GAP 43 from the soma to the end of each axon by using Fluoview 5.0 software (Olympus Corporation) and averaged.

3 Results and discussion

HFMs of PAN and PEEK-WC were prepared by dry–wet spinning technique in order to develop an in vitro tissue engineered system which allows hippocampal neuronal outgrowth.

Owing to its amorphous character and solubility in common solvents, PEEK-WC can be used for preparing membranes with different properties by an inexpensive and flexible method [23]. Previous studies demonstrated that PEEK-WC membranes in flat and HF configuration are able to support the metabolic functions of cells (e.g. hepatocytes, lymphocytes) [24, 25]. On the other hand PAN is one of the most used synthetic materials for the production of dialyzer membranes [26].

Although the biocompatible properties of both, PEEK-WC and PAN membranes, are well documented, information about their interaction with hippocampal neuronal cells is still lacking.

In this work towards the in vitro reconstruction of a biohybrid hippocampal tissue-engineered model the aim was to determine whether PAN HF and PEEK-WC HF membranes are able to allow not only a good cellular adhesion, but also the axonal outgrowth and differentiation of neuronal cells.

For culturing neuronal cells it is necessary to take in account that cell growth is strictly influenced by environmental cues. As consequence, to design a scaffold that is suitable for neuronal tissue engineering, a substrate must exhibit surface properties that elicit neuronal cell growth and functions. Therefore the developed PAN HF and PEEK-WC HF membranes have been fully characterized in terms of physico–chemical properties through dynamic water contact angle measurements giving important information about the wettability of each investigated surface. Before seeding out the cells both kinds of HFMs were coated with PLL in order to expose the cells to the same functional group and thus evaluating the cellular behaviour on homogenous surfaces since the intrinsic chemical characteristics are minimized by the coating.

The water contact angle measured before and after the modification displayed the strong effect on the physical–chemical properties exerted by the PLL coating.

Although PAN membranes keep their wettability character, both advancing and receding contact angles increased to values of 67,5° ± 4°and 43° ± 4°, respectively, after treatment with PLL (Fig. 1b) that results evidencing an increase of 12 and 15% compared to the values of the native substrate (Fig. 1a). On the other hand, in the case of PEEK-WC HF membranes the coating treatment led to a decrease of the advancing and receding contact angle values from 89° ± 3° to 77,6° ± 3° and from 67° ± 4° to 62.3° ± 5°, respectively (Fig. 1a–b). The coating effectively reduced the differences between the native surfaces as demonstrated by the advancing and receding contact angles which are expression of apolar and polar domains of the surface, respectively.

Since the permeability properties of the materials may influence axonal regeneration through the diffusion of

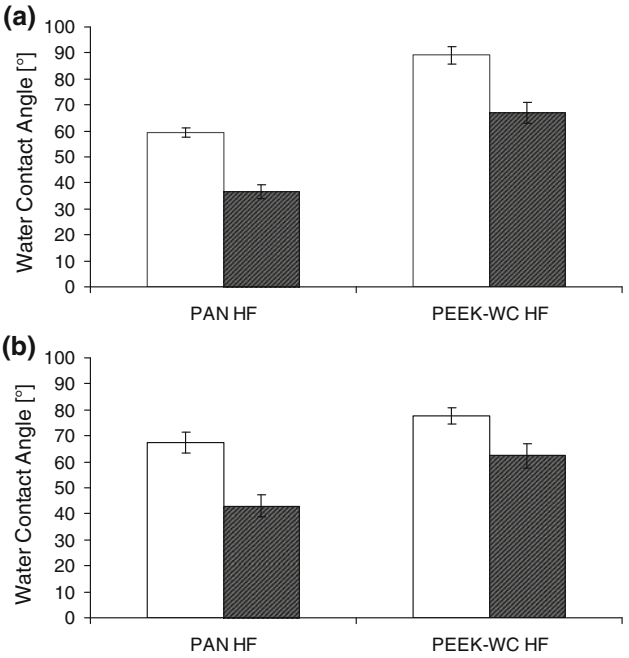

Fig. 1 Advancing (*white bar*) and receding (*black bar*) contact angle measured on: **a** native membranes and **b** PLL-coated membranes

nutrients and cell secreted soluble factors [27], in this work, experiments aimed to evaluate the hydraulic permeance of the HF membranes were carried out. The observed steady-state hydraulic permeance of the membranes, calculated as the slope of the flux (J) versus transmembrane pressure (ΔP^{TM}) straight line, was 0.239 l/h m^2, with an R-squared value of 0.98 for native PAN HF membranes and decreased after PLL coating at the values of 0.215 l/h m^2 (Fig. 2a). PEEK-WC HF membranes exhibited low hydraulic permeance with values of 0.089 and 0.030 l/h m^2, before and after PLL-coating, respectively (Fig. 2b). These results demonstrated that PAN HF membranes are more permeable with respect to PEEK-WC HF membranes and this is in good agreement with their more hydrophilic character as shown by contact angle measurements.

To investigate the potential application of such HFMs in neural tissue-engineering, neurons isolated from hippocampus were used. Polarization of these neurons occurs spontaneously in culture during the first 48–72 h, during which the neurons follow very well-defined stages, from stage 1 where cells are unpolarized, to stage 3 where the cells establish an axon [18]. Here, we investigated the neurite outgrowth of hippocampal neurons cultured on the outer surfaces of PAN HF and PEEK-WC HF membranes. As revealed by SEM analysis at DIV4 hippocampal neurons on the PAN HF membranes were trigonal pyramidal in shape with long neurites distributed along the fibers (Fig. 3a). At DIV8 it is evident a dense array of neurons

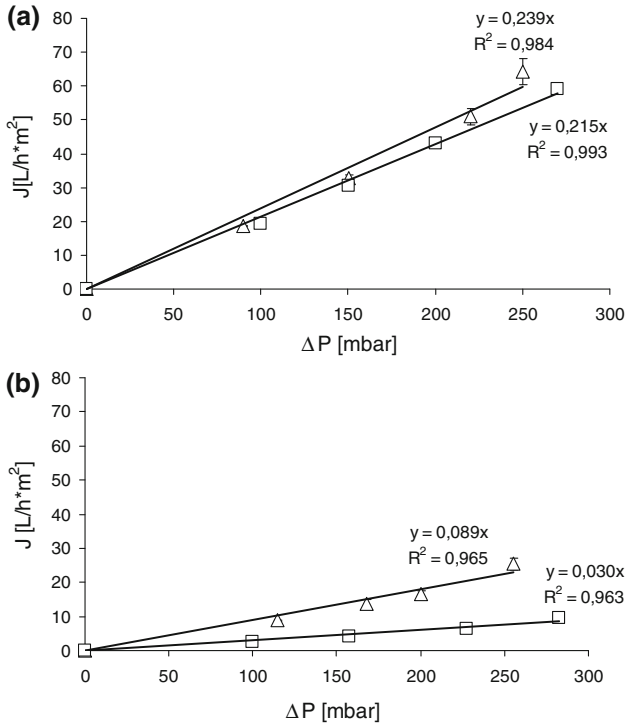

Fig. 2 Hydraulic permeation measurements of **a** PAN HF and **b** PEEK-WC HF membranes before (*upward triangles*) and after (*squares*) PLL-coating. Experimental values (*symbols*) were averaged on 10 measurements. The interpolation of experimental data is reported as a *solid line*

connected to each other via their axons and dendrites, extending along the fibers of PAN membranes and producing neuronal network which evidenced a three-dimensional structure (Fig. 3b).

The axon outgrowth increased with the increasing of the culture time and at DIV12 hippocampal neurons cultured on PAN HF membranes formed very complex neuronal network (Fig. 3c).

These results are comparable with those for hippocampal neurons that were cultured on PEEK-WC HF membranes at DIV4, DIV8 and DIV12, respectively (Fig. 3d–f).

The morphological observations revealed that primarily cultured hippocampal neurons were able to differentiate and to reach a neuronal network on the investigated HFMs, especially on PAN HF where more complex structures were formed. This is confirmed by morphometric analysis of axonal length at DIV8 and DIV12 (Fig. 4). Hippocampal neurons grown at DIV8 on PAN HF membranes extended axons that were significantly longer than those formed on PEEK-WC HF membranes, at the same developmental stage (133 ± 35 vs. 102 ± 15 μm. $P < 0.05$) (Fig. 4a–c). Axon lengths increased with time reaching the highest value of 147 ± 13 μm on PAN HFMs at DIV12.

The secretion of the neurotrophin BDNF that plays a critical role in the survival, differentiation and maintenance of neuronal cells [28], demonstrated that the cells adhering to the HF membranes were also functionally active as it is

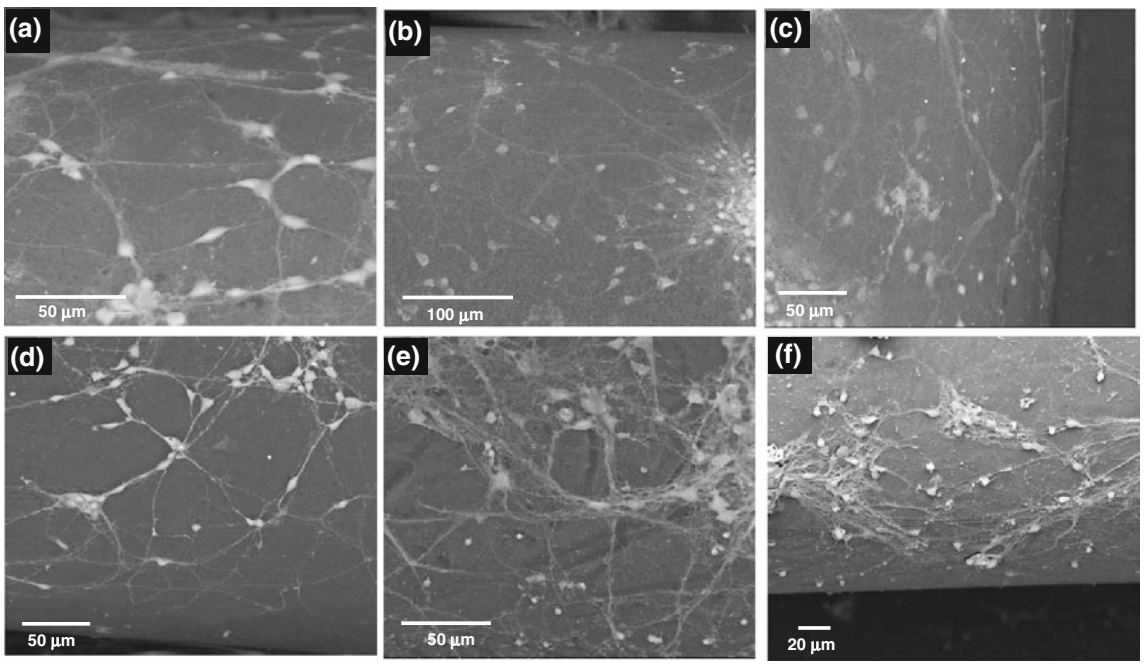

Fig. 3 SEM's images of hippocampal neurons at DIV4 (**a**, **d**), DIV8 (**b**, **e**) and DIV12 (**c**, **f**) on (**a–c**) PAN HF and **d–f** PEEK-WC HF membranes

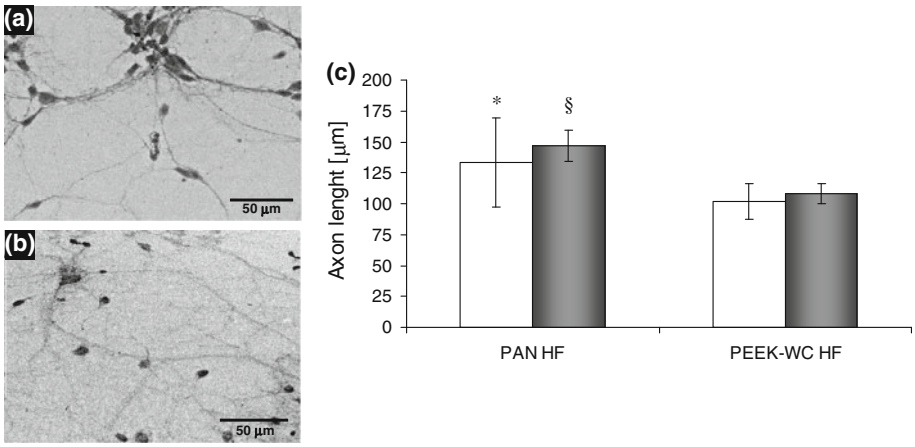

Fig. 4 Morphometric analysis of hippocampal neurons on PEEK-WC HF and PAN HF membranes. Images of neurons on PEEK-WC HF (**a**) and PAN HF (**b**) membranes. **c** Axon length of hippocampal neurons at DIV8 (*white bar*) and DIV12 (*grey bar*) on HFMs. The values were expressed as average ± standard deviation. Data statistically significant according to ANOVA followed Bonferroni *t*-test and *T*-Student's test. * $P < 0.05$ versus PEEK-WC HF at DIV8; § $P < 0.05$ versus PEEK-WC HF at DIV 12

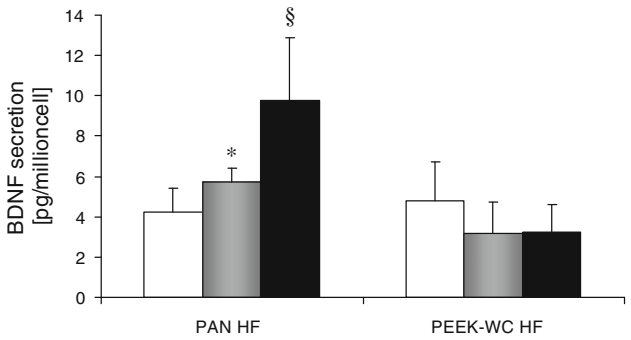

Fig. 5 BDNF secretion of hippocampal neurons at DIV4 (*white bar*), DIV8 (grey bar) and DIV12 (*black bar*) on PAN HF and PEEK-WC HF membranes. The values expressed as average ± standard deviation are the mean of six experiments and evaluated according to ANOVA followed by Bonferroni *t*-test and *T*-Student's test. * $P < 0.05$ versus PEEK-WC HF at DIV8; § $P < 0.05$ versus PEEK-WC HF at DIV12 and versus the same substrate at DIV4 and DIV8

shown in Fig. 5. In particular, on PAN HFMs the BDNF secretion increased with time, differently, on PEEK-WC HFMs the BDNF production decreased. Interestingly, the highest levels of BDNF were reached when neurons were cultured on PAN HFMs where at DIV12 values of 9.8 ± 3 pg/millioncell were revealed. Considering that BDNF is essential for neuronal survival and differentiation during development and for synaptic function and plasticity in the mature brain [29–31], these results demonstrated that PAN HFMs elicit neuronal functions and plasticity as confirmed by the correlation between BDNF secretion and axonal outgrowth. In fact, BDNF is distributed along both the dendrites and axons [32] where it induces the delivery of GluR2 which is a major receptor subunit involved with synaptic activation, the formation of hippocampal extrasynaptic spines and dendritic elongating processes [10, 33]. Moreover, it has been demonstrated that the immunoneutralization of BDNF is correlated with a reduction in GAP-43 levels and with a consequent decrease of neurite extension [34] performing a fundamental role in the control of the regenerative response.

Taken together the results obtained in this study demonstrated that primary hippocampal neurons respond to the different HFMs by regulating their morphology and changing their axonal growth and metabolic behaviour. The regulation of these responses is complex and depends on the properties of membranes. In particular, we suggest that the high hydraulic permeance of PAN HFMs seems to favour an axonal sprouting and growth enhancing the exchange of nutrients and cell soluble-secreted factors between cellular and extracellular compartments. Although the general architecture appeared to be independent of the membrane properties, the high permeability of the membranes facilitated axonal regeneration, in agreement with other studies [27]. In particular, it is well known that the permeability of the guidance conduit confines the molecular exchange between the enclosed transplant and the exterior wound environment. Even if the permeability is too high, it may allow inhibitory molecules to enter the substrate and inhibit axonal regeneration [35]. In vivo a selective permeability is desired to allow for free transport of nutrients and waste products across the conduit, while excluding large immunogenic or inhibitory molecules and host scar-forming cells from entering the conduit lumen. The results of this study indicate that PAN HFMs have hydraulic permeance that elicits neuronal outgrowth and differentiation since this membrane property enhances the mass transfer of nutrients and metabolites to the cells and the removal of catabolites.

Overall, the correlation of the membrane parameters with cellular responses is an important step toward the development of valuable biomaterials for neuronal tissue-engineering.

4 Conclusions

PAN HF and PEEK-WC HF membranes were developed and used for the culture of primary hippocampal neurons in order to realize biohybrid membrane systems which are able to create neuronal network in vitro. Results from this study indicate that both HFMs supported neuronal outgrowth, differentiation and the long-term maintenance of specific metabolic activities. These HFM systems offer valuable tools both for fundamental questions in neuroscientific research and a wide range of biotechnological applications.

Moreover, our findings interestingly demonstrated that the neuronal features in terms of axonal outgrowth and neurotrophin secretion reached the best expression levels and consequently induced the reconstruction of a more functional and complex neuronal network when neurons were cultured on PAN HFMs. These results strongly support the potential application of the PAN HF membrane system for the in vitro realization of a functional hippocampal tissue analogue to study neurobiological functions and/or neurodegenerative diseases. In particular, the differences in neuronal behaviour between the HF membrane systems suggest important roles for membrane-associated properties. This study confirmed that hydraulic permeance is an important parameter that must be considered in designing biomaterials in order to ensure adequate mass transfer conditions that are crucial for the neuronal architecture and functions.

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