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PREPARATION OF ORIENTED NEURONAL SUPPORTS

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Resumo

Um aspecto muito importante da regeneração nervosa é a recuperação da função após uma grave lesão que é, em geral, parcial ou insatisfatória. A aplicação de biomateriais em forma de tubos para criar uma ligação entre as extremidades do nervo danificado e um ambiente propício para regeneração é a técnica usada pelos investigadores. A utilização de tecidos de outras partes do corpo é a melhor estratégia para regeneração nervosa, contudo apresenta muitos inconvenientes. Como alternativa os investigadores têm-se debruçado na pesquisa de materiais sintéticos biodegradáveis. Estes materiais podem ser manipulados de várias formas com o objectivo de obter as propriedades mais adequadas para alcançar os melhores resultados na regeneração nervosa. Estes podem ser produzidos com diferentes dimensões, taxas de degradação, propriedades mecânicas, composição química e micro-arquirecturas (diferentes texturas como por exemplo microsulcos).

O objectivo deste trabalho centrou-se em investigar se a casca de camarão desproteinizada (Modified Exoskeleto of Shrimp (MES)) era um bom material para ser utilizado na regeneração nervosa. A MES possui uma forma côncava o que permite facilmente o seu enrolamento em torno das extremidades de um nervo danificado. Após a caracterização da MES, realizaram-se duas experiências distintas, uma para avaliar a biocompatibilidade da mesma e uma segunda para estudar o comportamento das células sobre os microsulcos produzidos na parte interna da MES. Nas experiências, utilizou-se células PC12, uma linha celular derivada da medula adrenal de ratos onde se formou um feocromocitoma (tumor). A adesão e proliferação das células sobre as MES foram parâmetros avaliados através de ensaios de MTT. A morfologia das células PC12 foi analisada recorrendo-se à microscopia electrónica de varrimento.

Os resultados obtidos demonstraram que as células PC12 aderiram e proliferam sobre as MES. O número de células aumentou ao longo do tempo de cultura tanto na camada interna como na externa das MES. As imagens de SEM obtidas mostraram que as células PC12 aderiram em toda a superfície das amostras apresentando uma forma arredondada. Também se verificou que a morfologia das células não depende da superfície onde as células foram semeadas. Através das imagens de SEM verificou-se que os microsulcos produzidos nas MES, com uma largura inferior a 10µm, não permitiram o crescimento direccionado das células PC12. No entanto, estes microsulcos permitiram o crescimento de neurites formadas pelas PC12 em diferenciação.

Em conclusão, os resultados apresentados demonstraram o potencial benefício na utilização de MES, com microsulcos, na regeneração de tecido nervoso.

Abstract

An aspect very important of nervous regeneration is the recovery of function after a serious lesion that is, in general, partial or unsatisfactory. The application of biomaterials in the form of conduits to create a bridge between the extremities of the injured nerve and an adequate environment to regeneration is the technique used by researchers. The utilization of tissues of another part of the body is the best strategy for nerve regeneration, however it presents many drawbacks. As alternative, the investigators have been looking for biodegradable synthetic materials. The materials can be manipulated of several forms with the aim of obtaining the properties more adequate to achieve the best results in nervous regeneration. They could be fabricated with different dimensions, degradation rates, mechanical properties, chemical composition and micro-architecture (different textures for example microgrooves).

The aim of this work is focused in investigating if the deproteinized shrimp shell (Modified Exoskeleton of Shrimp (MES)) was a good material to be utilized in nerve regeneration. The MES have a concave form that allows easily their winding around the extremities of injured nerve. After the characterization of MES, two distinct experiences where developed. The first one, to evaluate the biocompatibility of MES. The second, to study the behavior of cell cultures over produced microgrooves in the inner layer of MES. The experiments were performed with the pheochromocytoma PC12 cell line. The adhesion and proliferation of cells over MES were evaluated through MTT assays. The morphology of PC12 cells was analyzed using scanning electron microscope (SEM).

The results obtained demonstrated that PC12 cells adhered and proliferated over the MES. The cell number increased along the culture time, either in inner layer as in the outer layer of MES. The SEM images showed that PC12 cells adhered in all surface of samples, also presenting a similar morphology when cultured in the inner or

outer surface of MES. The produced microgrooves, with a width smaller then 10μm, where insufficient to allow the aligned growth of PC12, as observed in SEM images. Although, these microgrooves permit the alignment of small neurites formed in PC12 cells.

In conclusion, the results presented in this work showed evidences of the potential benefits of using MES, with microgrooves, in nervous regeneration.

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Abbreviations

BDNF	Brain derived neurotrophic factors
CNS	Central nervous system
COC	Copolymer 2-norbornene ethylene
CS	Chitosan
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulphoxide
DRG	Dorsal root ganglia
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ePTFE	Expanded polytetrafluoroethylene
FBS	Fetal bovine serum
FGF1	Fibroblast growth factor 1
Fk	Forskolin
GDNF	Glial-derived neurotrophic factor
HMDS	Hexamethyldisilazane
MES	Shrimp Modified Exoskeleton
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5,diphenyltetrazolium
NGF	Nerve growth factors
PBS	Phosphate buffered saline
PCL	Polycaprolactone
PCLEEP	Poly(caprolactone-co-ethyl ethylene phosphate)
PCLF–PPy	Polycaprolactone fumarate and polypyrrole
PDLA	Poly(D,L-lactic acid)
PDLLA/CS/CHS	PDLLA/Chondroitin sulfate/Chitosan

PDLLA/CS/CHS/NGF	PDLLA/Chondroitin sulfate/Chitosan/NGF
PGA	Poly(glycolic acid)
PHEMA	Poly(2-hydroxyethyl methacrylate)
PLA	Poly(lactic acid)
PLGA	Poly(lactide-co-glycolide)
PLLA	Poly(L-lactic acid)
PNS	Peripheral nervous system
Ppy	Polypyrrole
PVDF	Poly(vinylidene fluoride)
rhbFGF	Recombinant human basic fibroblast growth factor
SC	Schwann cells
SEM	Scanning electron microscope
8CPT-2Me-cAMP	8-(4-chloro-phenylthio)-20-O-methyladenosine-30,50-cyclic

I. Introduction

The major causes of nerve injury are motor-vehicle accidents, followed by sports-related accidents, falls and violence injuries (Hejcl *et al*, 2008). In the entire world, there are many people that suffer of nervous injury, both the central nervous system (CNS) (tetraplegia or paraplegia) and in peripheral nervous system (PNS) (Hejcl *et al*, 2008; Huang *et al*, 2006).

The most severe and challenging nervous problem to treat is the complete transection of a nerve (Haile *et al*, 2007). First, to avoid the lack of function, created by the injury, one should prevent the axons from dying. Also, the sprouting axons must extend from proximal nerve stump toward their target into the distal nerve stump, and establish synaptic connections to the sure target regions (Horner *et al*, 2000; Tabesh *et al*, 2009). So, successful regeneration depends upon the ability of injured axons to survive, re-grow, and reconnect with their original targets, processes integral to normal development (Bandtlow, 2003).

The efforts to attempt bridge nerve gaps go back to 1880 when Gluck used decalcified bone to bridge gaps (Taras *et al*, 2008; Gluck, 1880). Before World War II, many researchers have used various biological and non-biological materials in order to try to regenerate the nervous system, but nobody was recognized (Buenger, 1891; Formatti, 1904; Hudson *et al*, 2000; Nageottte, 1915; Platt, 1920; Taras *et al*, 2008). However, after World War II, arose a new interest and research by finding and experimenting more ambitious techniques, such as, autogenous blood vessels and synthetic materials to nerve repair (Taras *et al*, 2008).

The most important is to create the suitable conditions for axonal regeneration (Huang *et al*, 2006; Schmidt *et al*, 2003). Several tissue engineering strategies and scaffolds have been tried to create an ideal scaffold to bridge nerve gaps in comparison to the autografts (Huang *et al*, 2006; Schmidt *et al*, 2003; Tabesh *et al*, 2009). Although, the autografting is the best strategy to repair injured nerves, because it will not cause severe tissue reaction during the implantation period (Runge *et al*, 2010; Schmidt *et al*, 2003; Tabesh *et al*, 2009). However, it includes the difficulty of acquiring a donor nerve for grafting and the inevitable risks of surgery at another site (Runge *et al*, 2010; Schmidt *et al*, 2003; Tabesh *et al*, 2009). The use of an artificial implant has the advantage that it possible modifies their properties in a controlled fashion (Gu *et al*, 2011; Huang *et al*, 2006; Schmidt *et al*, 2003). So, it can be obtain a scaffold with excellent mechanical properties and an enhanced surface area to promote the proliferation of the support cells and axonal regeneration (Huang *et al*, 2006; Tabesh *et al*, 2009).

1.1. Anatomy of nervous system

The nervous system is consisting by the central nervous system (CNS) and the peripheral nervous system (PNS) (Tabesh *et al*, 2009). The CNS is constituted by the brain and spinal cord (Tabesh *et al*, 2009). Dendrites, axons, and cell bodies are the components that constitute the spinal cord (Schmidt *et al*, 2003). The center of the spinal cord, a butterfly-shaped region referred to as gray matter, contains the cell bodies of excitatory neurons, as well as glial cells and blood vessels (Dalton *et al*, 2008; Schmidt *et al*, 2003). The white matter surrounds the gray matter and help to protect and isolate the spinal cord (Schmidt *et al*, 2003). White matter is formed by axons and glial cells, as well as oligodendrocytes, astrocytes, and microglia (immune cells) (Dalton *et al*, 2008; Schmidt *et al*, 2003). The axons in the CNS are myelinated by oligodendrocytes (Schmidt *et al*, 2003).

The peripheral nervous system consists of nerve endings in the peripheral nerve trunks, plexuses and ganglia that connect the central nervous system with other body parts (Dalton *et al*, 2008; Kiernan *et al*, 2009; Schmidt *et al*, 2003). The peripheral axons are myelinated by Schwann cells forming Ranvier nodes (Dalton *et al*, 2008; Kiernan *et al*, 2009; Schmidt *et al*, 2003).

There is a strict relationship between the CNS and the PNS because the peripheral nerves contact with the muscles giving sensory and excitatory stimuli to and from the spinal cord and the CNS interprets these signals and provides excitatory stimuli to the PNS (Haile *et al*, 2007; Schmidt *et al*, 2003).

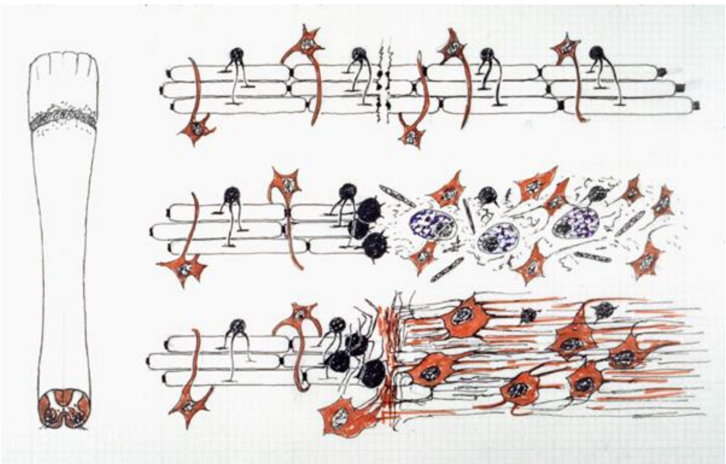



Figure 1. Formation of glial scar in the CNS. In the CNS, the few neurons that survive axotomy attempt regeneration and subsequently meet an impenetrable glial scar composed of myelin and cellular debris, as well as astrocytes (orange), oligodendrocytes (small black cells), and microglia. Fibroblasts, monocytes, and macrophages (purple) may also be present in the glial scar. Consequently, regenerating neurons in the spinal cord are blocked from reaching their synaptic target [1].

The major difference between the PNS and CNS is the capacity of first to regenerate after an injury (Dalton *et al*, 2008; Schmidt *et al*, 2003). The glial and extracellular environment of an injury in CNS has inhibitory influences in axonal regeneration and these environment cause a nearly impossible axonal regrowth (Bahr *et al*, 1994; Kiernan *et al*, 2009). Myelin-associated inhibitors of neurite growth, astrocytes, oligodendrocytes, oligodendrocyte precursors, and microglia migrate to the injury site making the environment nonpermissive for axonal growth (Dalton *et al*, 2008; Kiernan *et al*, 2009). In the distal stump inhibitory myelin and axonal debris are not removed readily because the degeneration is much sluggish in the CNS than in the PNS (Kiernan *et al*, 2009). As result it is formed a glial scar, due to the glial reaction that surround the axons (figure 1). Therefore neurons cannot regenerate and the axonal growth stops (Dalton *et al*, 2008; Kiernan *et al*, 2009; Silver *et al*, 2004).

The process of CNS regeneration has multi-steps, initially the injured neurons must survive and later they must extend their cut processes to its original neuronal targets (Aguayo *et al*, 1987; Bray *et al*, 1987; Dalton *et al*, 2009; David *et al*, 1987; Keirstead *et al*, 1989; Tabesh *et al*, 2009; Villegas-Perez *et al*, 1988). Then the axons regenerated need to be re-myelinated and form functional synapses with the surface of the targeted neurons (Bray *et al*, 1987; Dalton *et al*, 2009; David *et al*, 1987; Horner *et al*, 2000; Kiernan *et al*, 2009; Tabesh *et al*, 2009; Villegas-Perez *et al*, 1988).

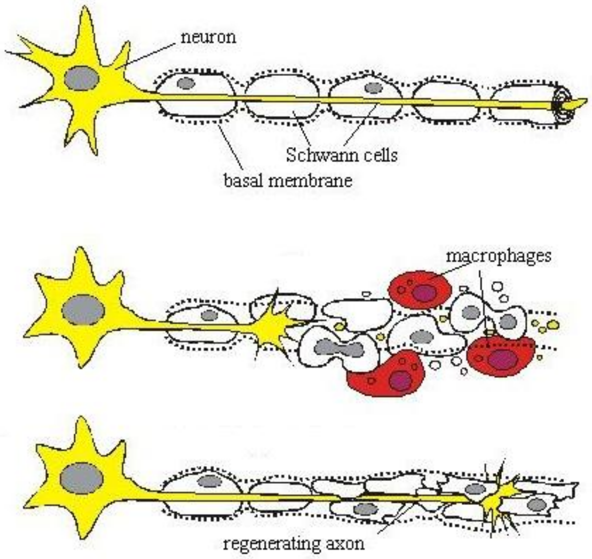


Figure 2. Peripheral nerve degeneration. In the PNS, support cells aid neuronal regeneration. Proliferating Schwann cells, macrophages, and monocytes work together to remove myelin debris, release neurotrophins, and lead axons toward their synaptic targets, resulting in restored neuronal function [2].

In the PNS, at the site of nerve damage, the distal portion of the axons begins to degenerate, due protease activity and separation from metabolic resources of the nerve cell bodies (figure 2) (Dalton *et al*, 2009; Schmidt *et al*, 2003). The cytoskeleton of axons starts to breakdown and then the cell membrane is disintegrated (Dalton *et al*, 2009; Haile *et al*, 2007; Heath *et al*, 1998; Schmidt *et al*, 2003). After the degradation of the cytoskeleton and membrane, Schwann cells surround the axons in the distal end shed (Dalton *et al*, 2009; Schmidt *et al*, 2003). Macrophages and Schwann cells that are phagocytic cells, remove myelin and axonal debris (Dalton *et al*, 2009; Fu *et al*, 1997; Haile *et al*, 2007; Schmidt *et al*, 2003; Stoll *et al*, 1989). Following debris clearance, regeneration begins at the proximal end and continues toward the distal stump (Dalton *et al*, 2009; Schmidt *et al*, 2003). New axonal sprouts usually emanate from the nodes of Ranvier that are nonmyelinated areas of axons located between Schwann cells (Kiernan *et al*, 2009; Schmidt *et al*, 2003).

1.2. Strategies for nerve regeneration

In the regeneration of nervous system, the processing is based in the direct end-to-end surgical reconnection of the ends of lacerated nerve (figure 3) or the application of a graft (Bozkurt *et al*, 2009; Huang *et al*, 2006). The first process allows the repair of small gaps or defects in the damaged nerve (Bozkurt *et al*, 2009; Huang *et al*, 2006). However, for a longer gap, large than 5 mm in length, is used a nerve graft (Taras *et al*, 2008; Wang *et al*, 2011).

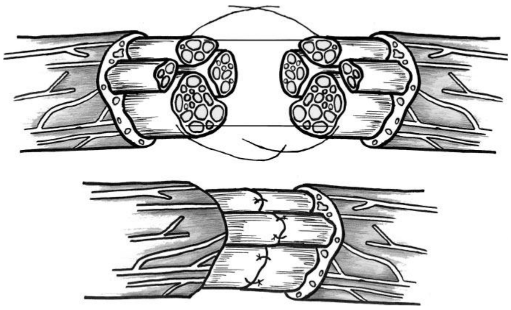


Figure 3. The suturing of individual fascicles within the nerve cable (Schmidt *et al*, 2003).

It is very important to create a permissive environment for the regeneration after the nervous system has been damaged (Schmidt *et al*, 2003). Many efforts have been made to create either natural or synthetic tubular nerve guidance channels (Johnson *et al*, 2008; Tabesh *et al*, 2009). The nerve conduits are used with the aim of mimic the three-dimensional and biological environment needed for enhanced regeneration (Tabesh *et al*, 2009). The conduits allow ward off external inhibitory molecules, preserve neurotrophic and neurotropic communications between the ends of the lacerated nerve and provide physical guidance for the regeneration of axons protecting from infiltration of fibrous tissue (Huang *et al*, 2006; Schmidt *et al*, 2003; Tabesh *et al*, 2009).

An ideal nerve guide conduit must have a set of desired physical properties such as: biodegradable and porous channel wall, the ability to deliver bioactive factors, the incorporation of support cells, an internal oriented matrix to support cell migration, intraluminal channels to mimic the structure of nerve fascicles and electrical activity (Huang *et al*, 2006; Liu *et al*, 2007; Sachlos *et al*, 2003; Schmidt *et al*, 2003; Tabesh *et al*, 2009)

To construct the nerve conduits several materials and techniques have been applied (Gu *et al*, 2011; Ni *et al*, 2010). The grafts could be of natural materials (autografts, allografts, xenografts and natural-based materials) or of synthetic materials (aliphatic polyesters such as poly(glycolic acid) PGA, poly(lactic acid) (PLA), polycaprolactone (PCL) and their copolymers (poly(lactide-co-glycolide) (PLGA) copolymer , poly(L-lactic acid) (PLLA)), electrically conducting polymers, including polypyrrole (Ppy), poly(phosphoesters) as poly(caprolactone-co-ethyl ethylene phosphate) (PCLEEP), polyurethanes, hydrogel-based such as poly(2-hydroxyethyl methacrylate) (PHEMA) and piezoelectric polymers as poly(vinylidene fluoride) (PVDF)) (Gu *et al*, 2011; Jiang *et al*, 2010; Ni *et al*, 2010).

1.2.1. Natural nerve graft

The natural nerve grafts could come from the same subject (autografts), from another subject of the same specie or cadaver (allografts) or from a different specie (xenografts) (Tabesh *et al*, 2009).

The "gold standard" of the natural nerve graft is the autograft, a segment of nerve removed from another part of the body of the person (Huang *et al*, 2006; Lundborg, 1988). Also autologous non-nerve tissues, such as veins, muscles, small intestine submucosa, tendons and epineurial sheaths, have been attempted as nerve grafts to bridge peripheral nerve gaps, showing limited success (Battiston *et al*, 2000; Brandt *et al*, 1999; Chiu *et al*, 1982; Geuna *et al*, 2000; Gu *et al*, 2011; Karacaoglu *et al*, 2001; Meek *et al*, 2002; Pagnotta *et al*, 2002; Risitano *et al*, 2002; Rodrigues *et al*, 2001; Tabesh *et al*, 2009; Walton *et al*, 1989). Natural tissue is biocompatible, less toxic and provides a support structure to promote cell adhesion and migration (Tabesh *et al*, 2009). This type of graft is associated with the disadvantages of necessity of a second surgical step to obtain the donor tissue, elimination of donor nerve function, a limited number of donors and the difference of dimensions between gap and graft (Chen *et al*, 2006; Jiang *et al*, 2010; Mackinnon *et al*, 1992; Ortiguela *et al*, 1987; Panseri *et al*, 2008; Runge *et al*, 2010; Tabesh *et al*, 2009).

Studies with autografts of PNS to bridge the spinal cord and the adult rat medulla oblongata after damage the CNS have showed that in a suitable environment the CNS axons can regenerate (David *et al*, 1981). In spite of the axons regrow, they didn’t re-enter in the host tissue (So *et al*, 1985). Other study that consists in graft an autologous PNS segment in the rat retina showed that axons of retinal ganglion cells grew and had the capacity to recognize target areas and re-establish functional synapses with target neurons (Aguayo *et al*, 1987; Keirstead *et al*, 1989).

Allografts and xenografts are available in large quantities which is a major advantage (Gu *et al*, 2011; Tabesh *et al*, 2009). However, they present disadvantages such as tissue rejection due to an undesirable immune response and some risk of disease transmission (Gu *et al*, 2011). The allogenic and xenogenic tissues must be processed to remove immunogenic components or must be submitted to immunosuppressive therapy (Gu *et al*, 2011; Mackinnon *et al*, 2001; Martini, 1994; Tabesh *et al*, 2009). The extracellular matrix (ECM) decellularized preserve and attempt mimic mechanically and physically the ECM of nerves helping regeneration (Gilbert *et al*, 2006; Gu *et al*, 2011; Whitlock *et al*, 2009). However, the ECM structure can be damaged or the extraction of the cellular components can fail in the process of decellularization, inducing inflammation upon implantation (Gu *et al*, 2011).

Crouzier and colleagues in 2008 decellularize a human umbilical cord artery and turned inside out to obtain a 3D scaffold with potential to be used as guidance channel. The structure was tested for resistance to collapse and cell growth capability (Crouzier *et al*, 2008). It was observed that the acellular scaffold possess comparable mechanical properties to natural nerves and that cells adhere and migrate through ECM (Crouzier *et al*, 2008).

In addition to the nerve graft of natural tissue, other natural-based materials have been tested for nerve repair. Some of these include fibrinogen, self-assembling peptide scaffolds, hyaluronic, alginate, agarose and chitosan, fibrin gels and ECM molecules (i.e. laminin, fibronectin and collagen) (Ahmed *et al*, 2000; Balgude *et al*, 2001; Grimpe *et al*, 2002; Haipeng *et al*, 2000; Hashimoto *et al*, 2002; Herbert *et al*, 1998; Holmes *et al*, 2000; Huang *et al*, 2006; Mosahebi *et al*, 2001; Seckel *et al*, 1995). Current studies are engaged in the modifications of these materials to enhance nerve regeneration and neurite extension (Dubey *et al*, 2001; Jiang *et al*, 2010; Leach *et al*, 2003; Sakiyama *et al*, 1999; Schense *et al*, 2000; Thomas *et al*, 2004).

The ECM molecules, such as laminin, fibronectin and collagen, are important in the development and growth of axons (Grimpe *et al*, 2002; Gu *et al*, 2011; Rutishauser, 1993). So, they have been applied as lumen fillers in nerve conduits in the form of hydrogels, channels, fibers, or porous sponges to serve as delivery vehicles for support cells, growth factors, or drugs (Gu *et al*, 2011).

The collagen is the material that has been further studied for nerve regeneration. Collagen-based microstructured 3D nerve guide containing numerous longitudinal guidance channels with dimensions resembling natural endoneurial tubes demonstrate direct the regenerated axons (Bozkurt *et al*, 2009). The nerve conduits have been performed predominantly on single channel conduits where the regenerated axons grow in a disperse way, resulting in inappropriate target reinnervation (Brushart *et al*, 1995; Yao *et al*, 2010). Yao and coworkers (2010) studied the influence of channels number in nerve regeneration using 1-, 2-, 4-, and 7-channel collagen conduits in rats with a 1 cm gap of sciatic nerve. The results showed that collagen conduits with 4 channels allow limited axonal dispersion and obtain similar quantitative results in nerve regeneration when compared to conduits with one channel (Yao *et al*, 2010). So, a multichannel nerve conduit with structural stability and favorable material and mechanical properties is needed to provide a reliable platform (Yao *et al*, 2010).

With the aim of enhance the axonal regeneration it has been developed strategies to incorporate cells or molecules in the nerve conduits (Tabesh *et al*, 2009). Schwann cells (SC), support cells, produce a number of growth factors that support the growth of axons, including nerve growth factors (NGF), brain derived neurotrophic factors (BDNF) among others (Alovskaya *et al*, 2007; Erschbamer, 2007; Tabesh *et al*, 2009). 2D and 3D nerve conduits of collagen allow SC to adhere and migrate throughout the guidance channels, forming cellular columns reminiscent of “Bands of Büngner” (Bozkurt *et al*, 2009; Chaudhry *et al*, 1992). The structures are crucial in the natural process of peripheral nerve regeneration during the Wallerian degeneration. (Bozkurt *et al*, 2009). Collagen sponge loaded with recombinant human basic fibroblast growth factor (rhbFGF) showed enhanced regeneration in rat sciatic nerve defects (Yao *et al*, 2007). Slowly degradation of collagen sponges achieved the long-term release of rhbFGF *in vivo*, improved the repair of sciatic nerve for a long period and finally, enhanced the sciatic nerve regeneration (Yao *et al*, 2007).

Gelatin, derived from denatured collagen, and other ECM molecules, such as laminin and fibronectin, were used in nerve scaffolds and mainly as lumen fillers of nerve conduits (Gu *et al*, 2011; Ijkema-Paassen *et al*, 2004). A study with adsorbed proteins (laminin) incorporated in microgrooves of poly(D,L-lactic acid) (PDLA) demonstrate that laminin improve adhesion of SC to the grooves (Miller *et al*, 2001). Gelatin-based scaffolds can incorporate bioactive cues that are gradually released during biomaterial degradation (Chen *et al*, 2004; Gu *et al*, 2011; Hanthamrongwit *et al*, 1996; Laemmel *et al*, 1998).

Wood and colleagues (2009) evaluated the effect of affinity-based delivery of glial-derived neurotrophic factor (GDNF) from a fibrin matrix in a nerve guidance conduit of silicone on nerve regeneration in 13 mm rat sciatic nerve defect. The histological results of this neurotrophic factor were compared to controls and NGF, demonstrating an increased maturity and organized architecture of the regenerating nerve under the influence of GDNF (Wood *et al*, 2009).

1.2.2. Artificial nerve grafts

An alternative to nature nerve grafts is the utilization of artificial materials to construct nerve conduits (Gu *et al*, 2011; Wang *et al*, 2011). These materials can be manufactured with different chemical composition, dimensions, mechanical properties, external geometries, degradation rates and micro-architectures to render them more ‘‘cell friendly’’ (Gu *et al*, 2011; Huang *et al*, 2006; Schmidt *et al*, 2003) Several studies have been made to achieve materials and methods that stimulate improved regeneration of nerve injuries (Jiang *et al*, 2010; Schmidt *et al*, 2003). The biomaterials applied could be non-degradable or degradable (Gu *et al*, 2011; Jiang *et al*, 2010; Wang *et al*, 2011).

1.2.2.1. Non-biodegradable materials

The non-biodegradable synthetic materials were the firsts to be tested before biodegradable materials (Braga-Silva, 1999; Gu *et al*, 2011; Lundborg *et al*, 1994; Stanec *et al*, 1998; Williams *et al*, 1983). Silicone rubber is the biomaterial traditionally used in nerve conduits for its elastic properties and because it is physiologically inert (Gu *et al*, 2011; Ni *et al*, 2010). But this material has various disadvantages characteristics such as being non-biodegradable, non-porous and needs a second surgery to be removed (Gu *et al*, 2011; Heath *et al*, 1998; Ni *et al*, 2010; Zhao *et al*, 1997).

Other non biodegradable material is the expanded polytetrafluoroethylene (ePTFE) (Braga-Silva, 1999; Gu *et al*, 2011; Lundborg *et al*, 1994; Stanec *et al*, 1998; Williams *et al*, 1983). Studies developed with this material showed similar results in functional motor and sensory recovery when compared with other autogenous or synthetic conduits, using gaps smaller than 4 cm (Wang *et al*, 2011; Stanec *et al*, 1998).

The problem associated to the non-biodegradable materials is that it remains in place as a foreign body, inducing a chronic foreign body reaction, with excessive scar tissue formation, limiting recovery of nervous function (Braga-Silva, 1999; Gu *et al*, 2011; Johnson *et al*, 2008; Merle *et al*, 1989). To overcome these disadvantages, researchers have been focused in the development of biodegradable materials (Gu *et al*, 2011; Huang *et al*, 2006; Runge *et al*, 2010; Taras *et al*, 2008; Thomas *et al*, 2004).

1.2.2.2. Biodegradable materials

Biodegradable materials have advantages compared to non-biodegradable materials, like preventing the formation of excessive scar tissue and a second surgery, to remove the material (Gu *et al*, 2011; Wang *et al*, 2011). They degrade within a reasonable time span, and the degradation products are absorbed by the body accompanied with mild foreign body reactions (Johnson *et al*, 2008; Gu *et al*, 2011). Moreover, the physiochemical and biological properties of biodegradable synthetic materials can be tailored to match different application requirements, and some chemical modifications enable the materials effectively to entrap support cells or bioactive molecules for controlled delivery during nerve regeneration (Gu *et al*, 2011; Hsu *et al*, 2009; Ni *et al*, 2009; Sill *et al*, 2008; Wang *et al*, 2011).

Several tissues engineering as well as cell therapies with support cells has been investigated for nerve regeneration (Gu *et al*, 2011). Schwann cells (SC), neural stem cells (NSC), embryonic stem cells, and marrow stromal cells are the more utilized among others (Gu *et al*, 2011).

Schwann cells were cultured over aligned poly-L-lactic acid (PLLA) fibers produced by electrospinning, aligned electrospun poly(e-caprolactone) (PCL) fibers and aligned electrospun PCL/gelatin biocomposite scaffolds in three different experiments (Chew *et al*, 2008; Ghasemi-Mobarakeh *et al*, 2008; Gu *et al*, 2011; Wang *et al*, 2009). In all studies the cells adhere to the biomaterial, grew and elongate neurites along the fibers produced (Chew *et al*, 2008; Ghasemi-Mobarakeh *et al*, 2008; Wang *et al*, 2009). The parameters of production of the fibers were manipulated to achieve the best conditions to improve the results of these scaffolds for nerve regeneration (Chew *et al*, 2008; Ghasemi-Mobarakeh *et al*, 2008; Morelli *et al*, 2010; Wang *et al*, 2009). In other study utilizing PLLA with grooves obtained by micro-patterned were verified the same results (Morelli *et al*, 2010).

Also to direct and enhance the growth of nervous cells have been tested biomaterials with microgrooves (Lin *et al*, 2008; Miller *et al*, 2001; Morelli *et al*, 2010). Materials as chitosan-gold (chi-Au) conduits, poly(L-lactic acid) (PLLA) membranes and substrates made of poly(D, L-lactic acid) (PDLA) have been target of experiments (Lin *et al*, 2008; Miller *et al*, 2001; Morelli *et al*, 2010). Reports have showed that the cells seeded over the referred materials adhere and grow preferentially along the grooves (Lin *et al*, 2008; Miller *et al*, 2001; Morelli *et al*, 2010). So, these materials guide the neurite extension and enhance their orientation creating a highly ordered neuronal cell matrix (Lin *et al*, 2008; Miller *et al*, 2001; Morelli *et al*, 2010). Also, there are evidences that the groove width influence SC alignment (Miller *et al*, 2001; Morelli *et al*, 2010). When these cells where cultured in PDLA with microgrooves, produced by compression molding and solvent-casting it was observed that SC were unable to adhere to materials with smaller microgrooves, especially if they were smaller than the size of the cells (Miller *et al*, 2001).

Several reports have been focused in the use of conductive biomaterials to stimulate the nervous cells to grow and elongate neurites enhancing nerve regeneration (George *et al*, 2005; Runge *et al*, 2010; Willerth *et al*, 2007). A material used for this is the polypyrrole (PPy) which is conductive and when incorporated in a biodegradable material creates a conductive biodegradable material (George *et al*, 2005; Mosahebi *et al*, 2001; Tabesh *et al*, 2009; Willerth *et al*, 2007). Runge and colleagues (2010) studied the behavior of PC12 cells and dorsal root ganglia (DRG) cultured over polycaprolactone fumarate and polypyrrole (PCLF–PPy). This experiment was developed to determine the optimal composition for both the electrical and biological properties (Runge *et al*, 2010). The results showed that PC12 cells grew over the biomaterial, developing elongated cell bodies and long neurites (Runge *et al*, 2010).

Apart of previously referred support cells other components could be incorporated in the biomaterials for nerve regeneration, like nerve growth factors (NGF) (Gu *et al*, 2011; Tabesh *et al*, 2009). They have the function of regulate cellular proliferation and differentiation (Gu *et al*, 2011). At the nerve injury sites they control the survival, proliferation, migration and differentiation of various cell types involved in nerve regeneration (Fu *et al*, 1997; Gu *et al*, 2011; Terenghi, 1999).

In an experiment made by Ni *et al* in 2009, bioactive molecules, including chitosan–nano gold (Au) and fibroblast growth factor 1 (FGF1), were successfully grafted onto PLA conduits by open air plasma treatment. The results demonstrated highest degree of myelination at both 4 and 6 weeks in PLA nerve conduits modified sequentially with chitosan–nano Au and FGF1 (Ni *et al*, 2009).

Xu and coworkers (2011) attempted to bridge a 10 mm defect in the siatic nerve of rats, using biodegradable PDLLA/Chondroitin sulfate/Chitosan (PDLLA/CS/CHS) nerve conduits prepared by immobilizing NGF in their lumen with carbodiimide PDLLA/CS/CHS/NGF. They have demonstrated that the use of nerve conduits prepared with nerve growth factors (NGF) enhanced the functional recovery when compared to PDLLA conduits (Xu *et al*, 2011). Also, the recovery could be similar to the one observed using autograft controls (Xu *et al*, 2011).

A novel poly(lactide-co-glycotide) (PLGA) microsphere-based spiral scaffold design with a nanofibrous surface was investigated and the results showed an enhanced surface area and possesses sufficient mechanical properties and porosities to support the nerve regeneration process (Valmikinathan *et al*, 2008). These scaffolds have an open architecture that goes evenly throughout the scaffolds hence leaving enough volume for media influx and deeper cell penetration into the scaffolds (Valmikinathan *et al*, 2008). The *in vitro* tests conducted using Schwann cells show that the nanofibrous spiral scaffolds promote higher cell attachment and proliferation when compared to contemporary tubular scaffolds or nanofiber-based tubular scaffolds (Valmikinathan *et al*, 2008). Also, the nanofiber coating on the surfaces enhances the surface area, mimics the extracellular matrix and provides unidirectional alignment of cells along its direction (Valmikinathan *et al*, 2008).

II. Objective

The aim of this report was to study the biocompatibility of modified exoskeleton of the shrimp (MES) for nervous tissue regenerations, using a nervous cell line PC12. Chitin and derivate Chitosan are biomaterials recovery from shrimp shells that have been reported useful for biomedical applications such as wound healing and dressings, drug delivery agents between other and also have the capacity to allow the guided growth of cells (Dutta *et al*, 2004; Teng *et al*, 2001).

Biocompatibility of MES was evaluated by analysis of viability/proliferation (MTT assay) and morphology (SEM). The two layers (inner and out) of this biomaterial were evaluated.

Further, using the inner face of MES, it was evaluated the influence of microgrooves in the cell behavior and differentiation. As in previous experience, it were evaluated the viability/proliferation of adherent cells and their morphology.

III. Materials and methods

3.1. Materials

All cell culture chemicals and supplies were purchased from Merck and Sigma-Aldrich (St. Louis, MO). All tissue culture flasks and plates were obtained from Corning (Corning, NY).

3.2. Characterization of material

The material used in the experiments was the shrimp shells. The shells were immersed in a solution of (3%) sodium hydroxide (NaOH) and heated to 70 ºC, with eight changes of solution to remove the proteins. This process was repeated until the samples presented a whitish aspect. Following, the shells were washed in deionized water, passing by an ultrasound machine during 15 minutes, to remove the remains of tissue and NaOH. These deproteinized shells shrimp - Shrimp Modified Exoskeleton (MES) – were, also, autoclaved and cut to size of the wells of 96-well plates with a punch.

The MES were maintained in standard culture conditions, Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco/BRL), penicillin-streptomycin (100 IU/mL and 10 mg/mL, respectively), and incubated in a humidified atmosphere of 5% CO_2 in air at 37ºC. The morphology of MES, both inner and outer surface, was evaluated by SEM.

To provide the direct orientation of the cell growth, microgrooves were created in the inner surface of the membranes. The microgrooves were performed with the help of a microhardness microscope (Shimadzu). The microscope has a pyramid-shaped diamond needle that is pressed into flat material samples with a small force (25 g). It was used the Vickers method and the hardness of the membrane was determined by measuring the diagonal of the indent obtained. The width of the microgrooves varies between 15 to 20 microns that is approximately the size of the cells. The evaluation and characterization of microgrooves were made also using the SEM.

3.3. Cell culture

PC12 cells, a pheochromocytoma cell line derived from the rat adrenal medulla, were donated by INEB (National Institute of Biomedical Engineering). Cells were maintained in standard culture conditions, Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco/BRL) and penicillin-streptomycin (100 IU/mL and 10 mg/mL, respectively), and incubated in a humidified atmosphere of 5% CO_2 in air at 37ºC. Culture medium was changed twice a week.

At 70 to 80% cell confluence, adherent cells were enzymatically released which consisted in the following steps: medium was removed and the cell layer was washed with phosphate buffered saline (PBS, at 37ºC), a solution of 0.04% trypsin in 0.25% EDTA (1 mL) was added to detach adherent cells, and the culture plates were incubated at 37ºC in a humidified atmosphere (5% CO_2 in air) for 10 minutes. Complete culture medium (2 mL) was added to the culture plates to stop the enzymatic reaction. The resulting cell suspension, at density of 10^5 cell/cm^2, was cultured over MES.

3.4. Experimental protocols

3.4.1. Evaluation of MES biocompatibility

To analyze MES biocompatibility, PC12 cells were cultured over MES and their behavior was observed for a period of 9 days. PC12 cells, at a density of 10^5 cell/cm^2, were cultured over MES membranes (inner and outer surfaces). Previously, MES were placed in 96-well culture plates and coated with 15 μg/cm^2 poly-L-lysine (Sigma) as showed in figure 4. Cells cultured on the standard tissue culture plates were used as control. The cell viability/proliferation was analyzed through MTT assay and the cell morphology was evaluated by SEM, at defined time points for 9 days culture time.

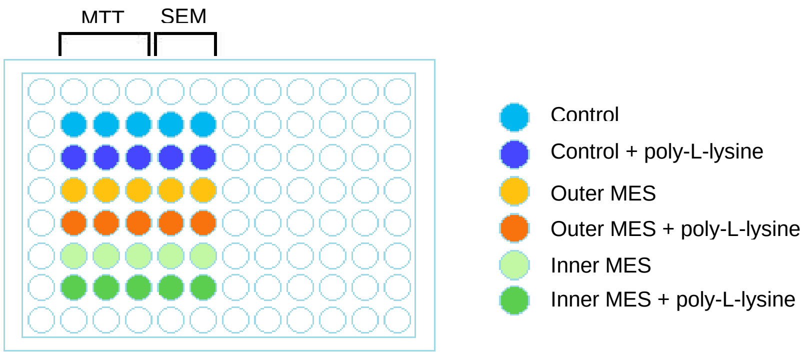


Figure 4. Schematic representation of cell culture. Cells were cultured over the MES, control, inner and outer surface without and with poly-L-lysine. The culture plate, as illustrated, was divided for two different analyzes, MTT assay and SEM, at defined points during 9 days.

3.4.2. Evaluation of cell orientation in produced microgrooves

Cells were cultured in 96-well culture plates, at the density of 10^5 cell/cm^2 over the inner surface of MES. Cells cultured in 3 cm culture plates were used as control.

The MES were previously placed in the plates as illustrated in the figure 5. The experiment was realized during 14 days. At the 10th day, at 70% cell confluence, 2.5% DMSO was added to the culture medium to differentiate the cells. The cell viability/proliferation was analyzed through MTT assay and the cell morphology was evaluated by SEM, at defined points over 14 days of culture.

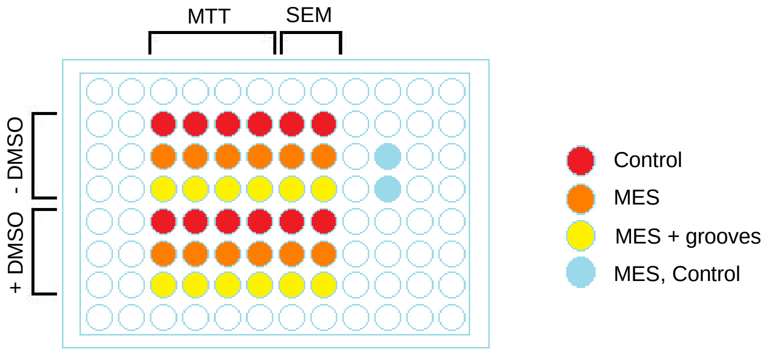


Figure 5. Schematic representation of cell culture. Cells were cultured over the plate, MES and MES with microgrooves. The culture plate, as illustrated, was divided to conduct two different analyzes, MTT assay and SEM, at defined points during 14 days. Experiments were performed in the absence and in the presence of DMSO, to differentiate PC12 cells. MES were also incubated with culture medium in the absence of cells (MES, control).

3.5. Cell viability/proliferation

Cell viability/proliferation of control and MES cultures was analyzed with MTT assay, at defined points.

Colorimetric assays based on tetrazolium dye reduction, as MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide), are extensively used in *in vitro* evaluation of viability, proliferation and activation of the cells. This assay is a quantitative method that consists in the ability of mitochondrial dehydrogenases, in live cells, to transform the MTT in a dark blue formazan crystals (Donaldson, 1998; Masters, 2000).

MTT (0.5mg/mL) was added to the culture and the culture plates were incubated for 3 h at 37ºC. After the incubation period, the culture medium was removed; the formazan salts were dissolved with 100 μL of dimethylsulphoxide (DMSO) and the absorbance was determined at λ = 550 nm on ELISA reader (Biotek, model Synergy HT). Results were expressed as absorbance (Abs).

3.6. Cell morphology

Evaluation the morphology and the spatial relationships of adherent PC12 cells over MES was performed with scanning electron microscopy (SEM). This method provides three-dimensional visualization at high resolution and the samples must first be chemically fixed, dehydrated, and coated with a conductive material (Bechara *et al*, 2010; Kaminskyi *et al*, 2008).

For the fixation process, the medium was removed and the samples were washed twice with PBS. Cells were fixed with 1.5% glutaraldehyde in 0.14 M sodium cacodylate buffer (pH 7.3), and left for 10 min. Glutaraldehyde was removed, and cells were washed twice with PBS. Cells were maintained at 4ºC in a solution of 0.14 M sodium cacodylate buffer (pH 7.3) (Bechara *et al*, 2010).

To prepare the samples for observation on SEM, the samples were dehydrated with increasing concentrations of ethanol (70%, 80%, 90% twice, and 100%) for 10 min each. Cells were further dehydrated with solutions of hexamethyldisilazane (HMDS) and ethanol, in the respective percentages (50%-50%, 60%-40%, 70%-30%, 90%-10% and 100% of HMDS) for 10 min each, in the hotte. After, the samples were left to dry in the hotte during one day (Bechara *et al*, 2010).

For SEM analyzes, the samples have, also, to be electrically conductive. So, samples were stick in a carbon ribbon, placed in an aluminum bracket and then covered with a thin layer of gold (Kaminskyi *et al*, 2008; Runge *et al*, 2010).

The samples were then analysed in a JeoL JSM 6301F scanning electron microscope equipped with a X-ray energy dispersive spectroscopy (EDS) microanalysis capability (Voyager XRMA System, Noran Instruments).

3.7. Statistical analysis

Data are presented as mean ± SEM of four replicas of each experiment.

Data analysis was performed using Mann Whitney U test. A p value of $p \leq 0.05$ was considered statistically significant.

IV. Results

4.1. Characterization of material

MES (inner an outer surface) were characterized using SEM and the results obtained are showed in figures 6 and 7.

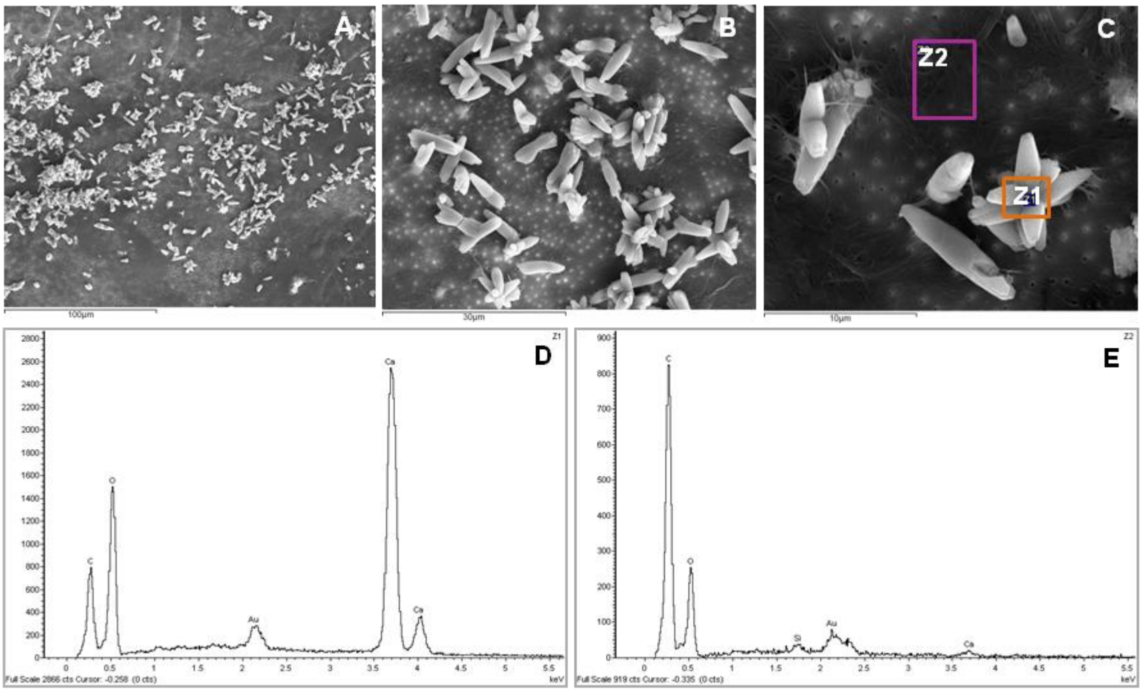


Figure 6. Morphology of the inner surface of MES – representative images of SEM.

(A) Aspect of inner surface of MES (magnification 500x);

(B) Detail of crystals of MES (magnification 2000x);

(C) High magnification of MES(magnification 5000x);

(D) Spectrum Z1 corresponding to the composition of crystals;

(E) Spectrum Z2 corresponding to the composition of surface of MES.

The inner layer (figure 6) presented a surface with small pores and great amount of crystals (figure 6 A, B and C). The major substance in this layer was carbon, as observed by analysis of spectrum (figure 6 E). The crystals present in the surface were composed of calcium carbonate (figure 6 D).

The outer layer of MES presented a great amount of carbon, has observed in the inner surface. But it was also observed some zones essentially composed by calcium phosphate and carbonate (figure 7 A, B and C).

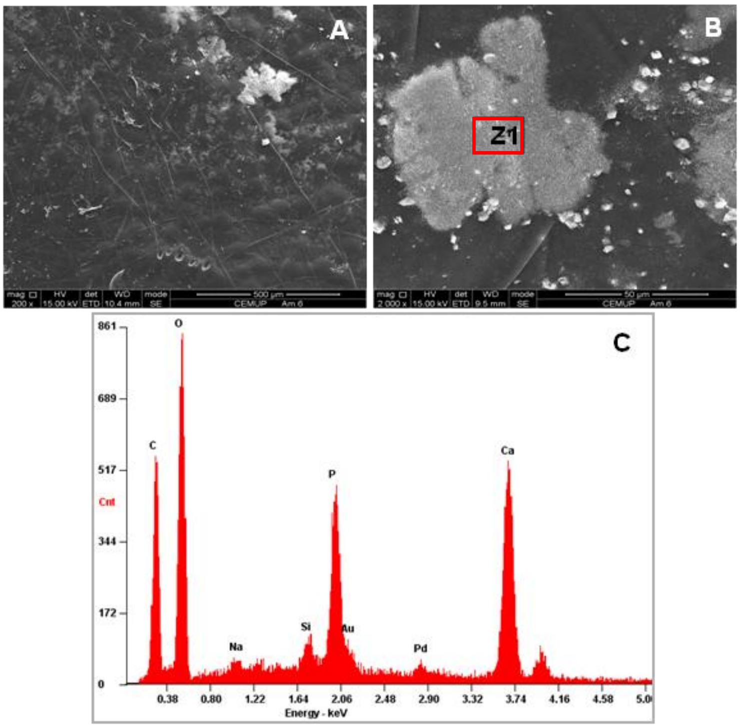


Figure 7. Morphology of outer surface of MES – representative images of SEM

(A) Aspect of outer surface of MES (magnification 200x);

(B) Marking of zone to do spectrum (magnification 2000x);

(C) Spectrum Z1 corresponding to the composition of the clear zone.

The characterization of the microgrooves created with the help of a microhardness microscope was also performed. The results are shown in figure 8.

In each MES sample, five microgrooves were created with a width between 15 and 20 microns (figure 8 A – y), which is approximately the size that PC12 cells need to adhere (Farina, 2008; Hoang *et al*, 2009; Spratt, 1998). The microgrooves created using the microhardness microscope were not the ideal ones, as observed in the figure 8 A and B. The diamond needle marked only small V-form microgrooves.

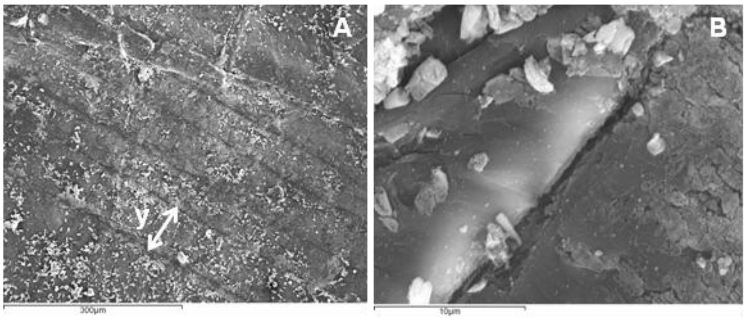


Figure 8. Microgrooves on the inner surface of MES – representative images of SEM.

(A) Aspect of the microgrooves obtained with the microhardness microscope. The microgrooves must have a width of y (magnification 200x);

(B) Detail of a microgroove (magnification 5000x).

4.2. Evaluation of MES biocompatibility

In a first step, a study was developed to evaluate the behavior of PC12 cell line cultured over MES. So, the cells were cultured over the inner and outer surface of MES and the cell viability/proliferation and morphology were analyzed by MTT assay and SEM respectively.

4.2.1. Cell viability/proliferation

Cell viability/proliferation was evaluated through MTT assay. Therefore in the MTT assay, viable cells reduce the MTT salt with the formation of a formazan precipitate, which is subsequently solubilized with DMSO. Results obtained are shown in figure 9.

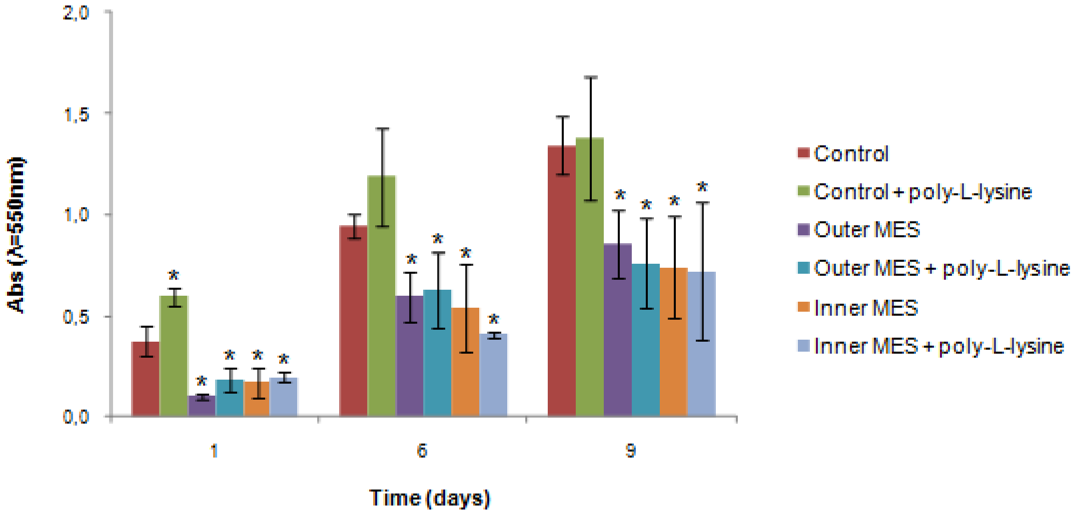


Figure 9. Cell viability/proliferation of PC12 cells cultured over the inner and outer surface of MES

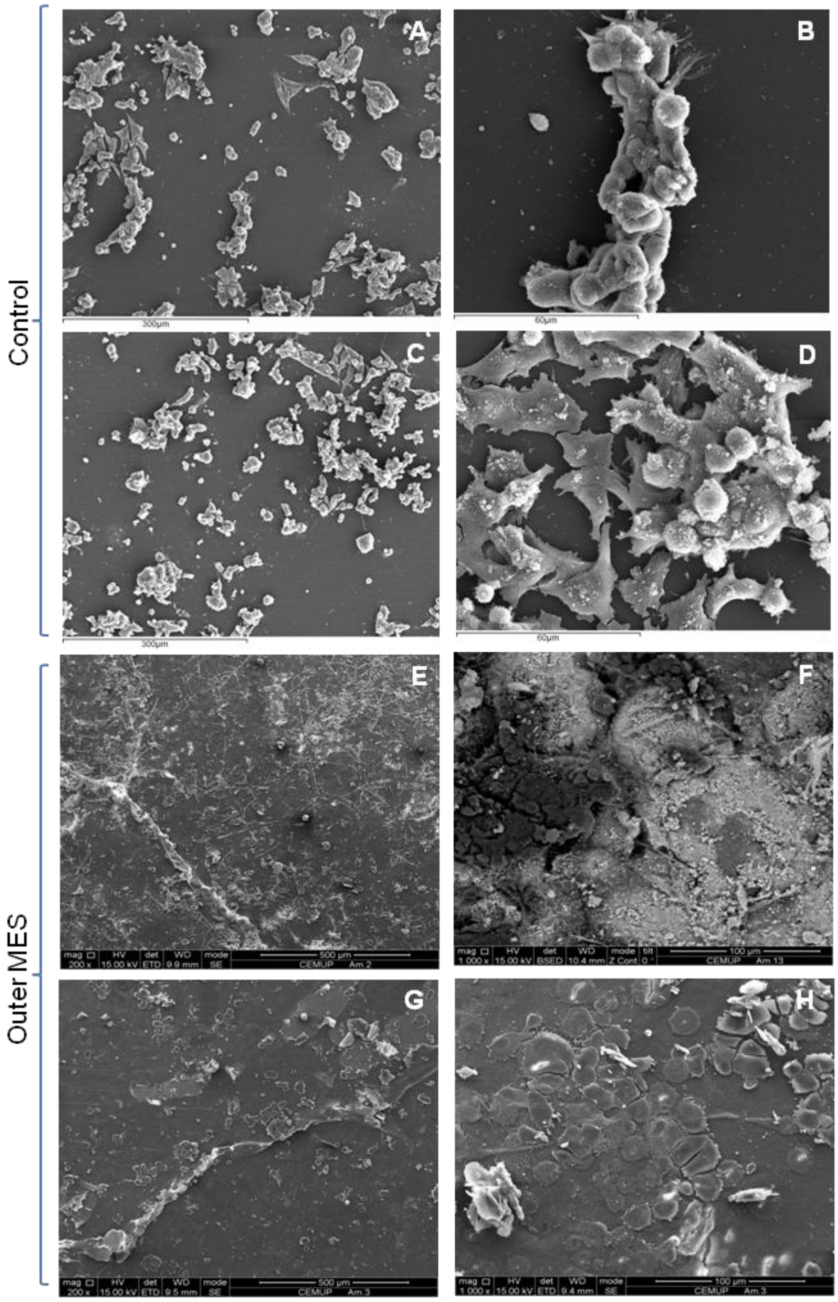
MTT assay throughout the 9 day culture time (cell density of 10^5 cells/cm^2).

*Significantly different from control (standard tissue culture plate).

Figure 9 shows the results observed in the MTT assay throughout the 9 days of culture time. Cell viability/proliferation increased during the time of the experiment in all samples (control, inner and outer membrane without and with poly-L-lysine). The control samples have an increased viability/proliferation when compared to the cultures over MES. Also, between the control cultures and control cultures with poly-L-lysine, it is possible to observe that the second has higher values in all days of the experiment. Observing the viability/proliferation of PC12 cultured over MES it was not possible to observe significant differences between the two layers, throughout the days of culture. Also, the presence of poly-L-lysine in the surface of MES did not influence the viability/proliferation of PC12, in opposite to the results observed in the control culture.

4.2.2. Cell morphology

The samples were, also, evaluated by SEM. The results are shown in figure 10.



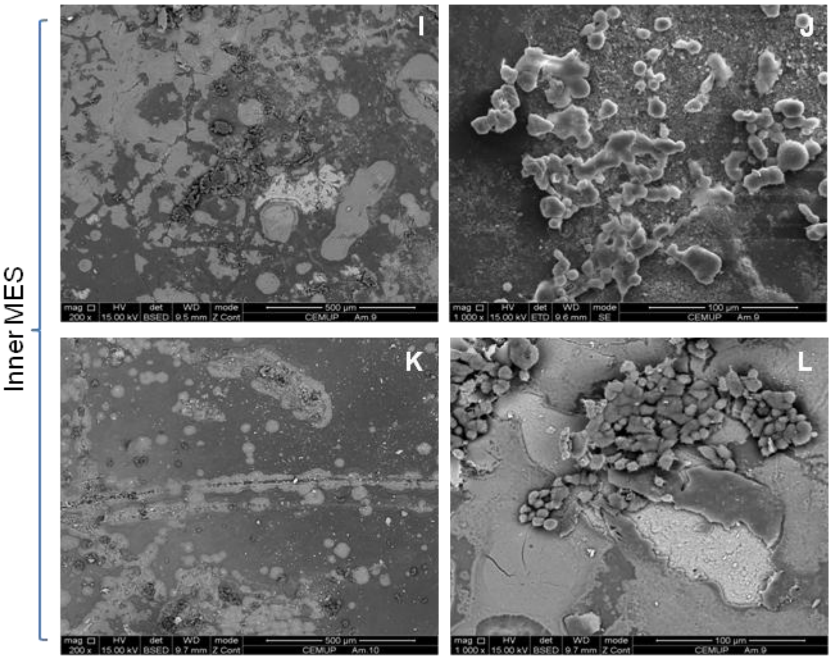


Figure 10. Morphology of PC12 cells cultured over MES at day 6 – representative images of SEM

(A) Control (magnification 200x);
(B) Control (magnification 1000x);
(C) Control with poly-L-lysine (magnification 200x);
(D) Control with poly-L-lysine (magnification 1000x);
(E) Outer MES (magnification 200x);
(F) Outer MES (magnification 1000x);
(G) Outer MES with poly-L-lysine (magnification 200x);
(H) Outer MES with poly-L-lysine (magnification 1000x);
(I) Inner MES (magnification 200x);
(J) Inner MES (magnification 1000x);
(K) Inner MES with poly-L-lysine (magnification 200x);
(L) Inner MES with poly-L-lysine (magnification 1000x).

Control cultures presented PC12 cells with a rounded morphology (figure 10 A, C), arranged in clusters or clumps. The presence of poly-L-lysine didn’t influence the morphology of the cells. The cells cultured on the surface of MES presented a similar morphology to control cultures (figure 10 A, C, J and L).

4.3. Evaluation of cell orientation in produced microgrooves

As have been documented in other works, nerve conduits with microgrooves allow to direct the neurites growth and consequently improved nerve regeneration through connection of nerve stumps. So, a second study was developed to investigate the influence of microgrooves in MES in the adhesion, proliferation and orientation of PC12 cells. Also, the differentiation of cells was evaluated and their subsequent orientation in the microgrooves.

4.3.1. Cell viability/proliferation

Cell viability/proliferation was evaluated through MTT assay. Therefore in the MTT assay, viable cells reduce the MTT salt with the formation of a formazan precipitate, which is subsequently solubilized with DMSO. Results obtained are shown in figure 11.

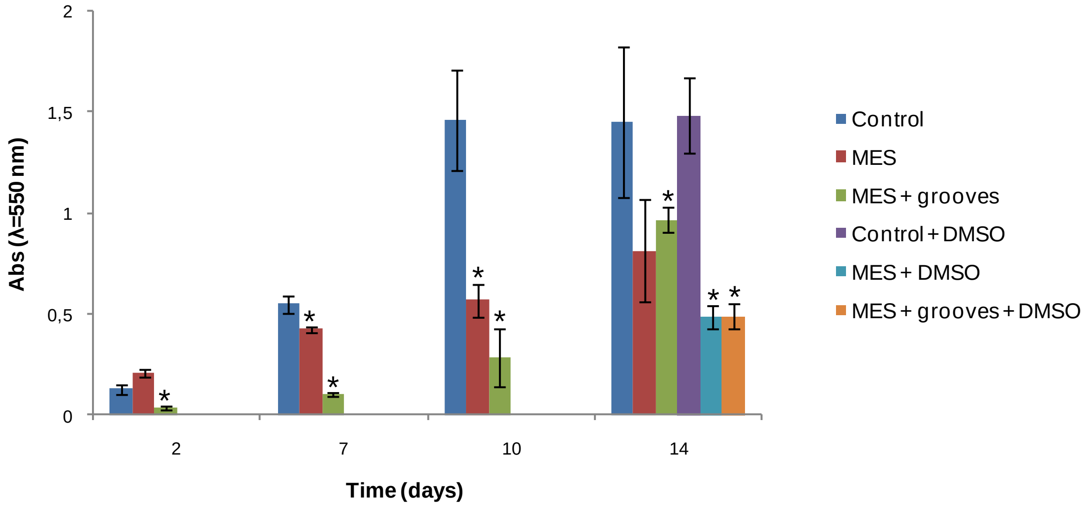


Figure 11. Cell viability/proliferation of PC12 cells cultured over inner surface of MES without and with microgrooves during all experience and without and with DMSO at day 14

MTT assay throughout the 14 day culture time (cell density of 10^5 cells/cm^2).

*Significantly different from control (standard tissue culture plate).

Figure 11 shows the results observed in the MTT assay throughout the 14 days of culture time. The viability and proliferation of control cultures increased over the culture period until day 10, as observed in figure 11. After this period, the culture kept a similar viability/proliferation value. Also, the addition of DMSO, at day 10 of culture period, did not affect the viability/proliferation of the cells. When cells were cultured in the inner layer of MES, their viability/proliferation was decreased compared to control. Also, the viability/proliferation of the cells cultured in the MES and with DMSO has also decreased between day 10 and 14.

4.3.2. Cell morphology

The samples were evaluated for alterations in their morphology, by SEM. The results are shown in figures 12 and 13.

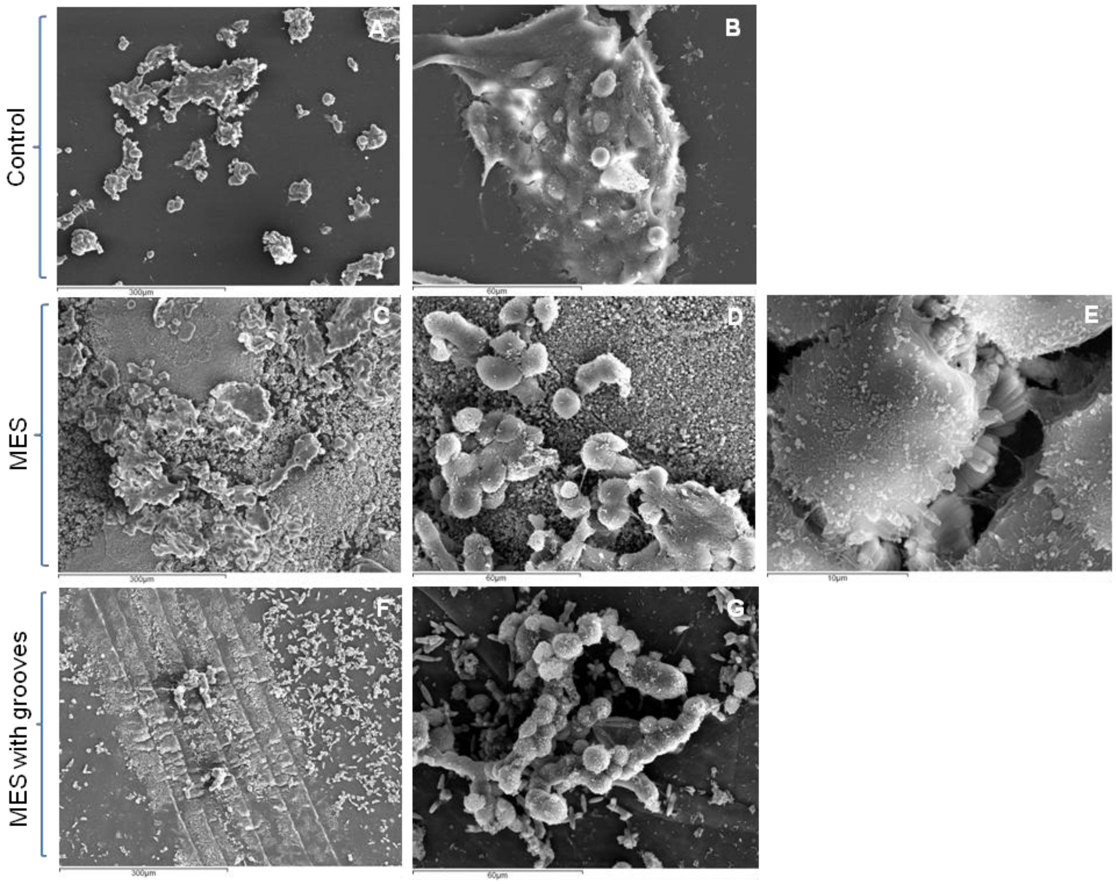


Figure 12. Morphology of PC12 cells without DMSO at day 14 of culture – representative images of SEM

(A) Control (magnification 200x);
(B) Control (magnification 1000x);
(C) MES (magnification 200x);
(D) MES (magnification 1000x);
(E) MES (magnification 5000x);
(F) MES with microgrooves (magnification 200x);
(G) MES with microgrooves (magnification 1000x).

Control cultures presented clusters of cells with rounded morphology, as observed in figure 12 A and B. After the addition of DMSO, it was possible to observe the presence of extensions, suggesting a differentiation of PC12 cells.

When cells were cultured in MES, the morphology was identical to control cultures (figure 12 C and D). Also, as observed in the figure 12 E, the cells where able to adhere to the crystals present in the surface. The addition of DMSO permitted the differentiation of PC12 cells, as observed in the figure 13 B, C, E and G. It was possible to observe cells with small extensions.

The microgrooves made in the inner surface of MES did not have the width necessary to cells to adhere and orientate along them (figure 12 and 13 (F and G)). So, the cells adhered aleatory in the surface of MES. Although, the results were not the expected ones, it was observed a possible oriented growth of the neurites that attached within the microgrooves (figure 13 G).

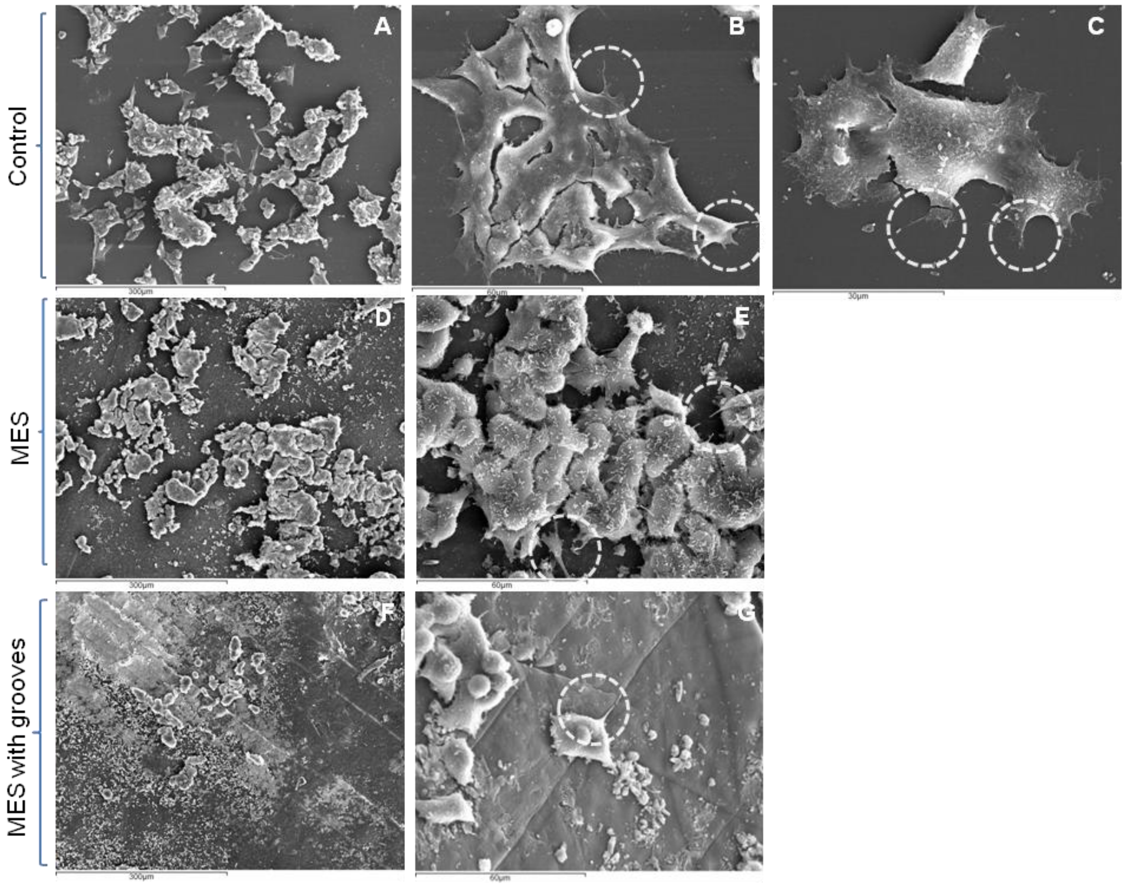


Figure 13. Morphology of PC12 cells with DMSO at day 14 of culture – representative images of SEM

(A) Control (magnification 200x);
(B) Control - the circles indicate the growth of neurites (magnification 1000x);
(C) Control - the circles indicate the growth of neurites (magnification 2000x);
(D) MES (magnification 200x);
(E) MES - the circles indicate the growth of neurites (magnification 1000x);
(F) MES with microgrooves (magnification 200x);
(G) MES with microgrooves - the circles indicate the growth of neurites (magnification 1000x).

The MES present some natural microgrooves, as observed in figure 14. In this case, the microgroove has the ideal lenght of 15.6 µm which permits PC12 cells to adhere and proliferate. The results showed that PC12 cells were able to grow in the same orientation of the microgroove.

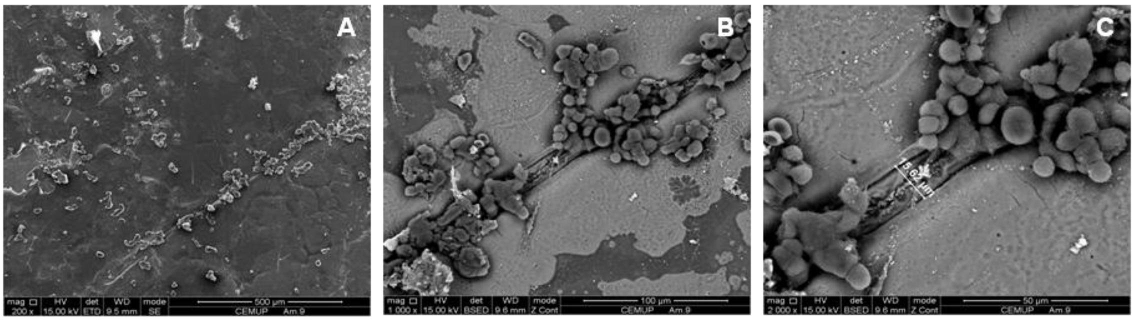


Figure 14. Width of a natural microgroove in MES – representative images of SEM

(A) Aspect of a natural microgroove (magnification 200x);

(B) Adhesion of cells in the microgroove (magnification 1000x);

(C) Marking of a width of a natural microgroove (magnification 2000x).

V. Discussion

Shrimp shells are composed by multi layer membranes of chitin with high content of calcium carbonate and phosphates (Heredia *et al*, 2007; Rodde *et al*, 2008).

Chitin is a naturally polysaccharide, the second more abundant, and is originated from exoskeletons of crustaceans and also from cell walls of fungi and insect (Jayakumar *et al*, 20010). Chitosan is the deacetylated derivative of chitin (Ishikawa *et al*, 2007; Pillai *et al*, 2009).

Chitin and chitosan (CS) polymers have unique structures, multidimensional properties, highly sophisticated functions and wide ranging applications in biomedical and other industrial areas (Chandy *et al*, 1990; Paul *et al*, 2000; Pillai *et al*, 2009; Muzzarelli *et al*, 2005). These materials present excellent biocompatibility, biodegradability and adsorption properties, low toxicity, low immunogenicity and are highly insoluble in aqueous solutions (Hirano *et al*, 1999; Jayakumar *et al*, 2007; Kurita *et al*, 2006; Mourya *et al*, 2008; Pillai *et al*, 2009; Rinaudo *et al*, 2008; Yi *et al*, 2005).

In this work, it was analyzed the use of shrimp shells in the regeneration of nervous system. *In vitro* analyses were performed using a neuronal cell line, PC12.

First, the composition and morphology of MES were analysed. The membranes obtained from the shrimp shells presented irregular surface morphologies. As observed in this study, the MES presented white spots and crystals of calcium carbonate, in the outer and inner surface respectively. It has been described by others authors that the presence of these structures is due to the treatments that they suffer, eg the frozen storage or the deproteinization (Heredia *et al*, 2007; Mikkelsen *et al*, 1997).

This work was divided in two different parts. A first step consisted in the evaluation of the compatibility of MES with the PC12 cells. The second part was to evaluate if the presence of microgrooves in MES influenced the cell behavior.

The cells used in the experiments were the PC12 cells. They are a cell line derived from a pheochromocytoma of the rat adrenal medulla and are small (5–10 μm) (Farina, 2008; Hoang *et al*, 2009; Spratt, 1998). The pheochromocytoma cell line has been widely used as a model of neuronal cell function because it exhibits many of the functions observed in primary neuronal cultures (Yue *et al*, 2009).

In the first experiment, the cells were cultured with a density of 10^5 cell/cm^2 over the inner and outer surfaces of MES during 9 days. Through a MTT assay, it was possible to observe that the viability/proliferation increased along the time of culture. PC12 cells proliferated more in control cultures (performed in standard tissue culture plates) than in the inner and the outer surfaces, both with and without poly-L-lysine. The control cultures with poly-L-lysine presented a higher viability/proliferation when compared to the other samples, in all tested days. However, in the inner and outer surface of MES, both with and without poly-L-lysine, the viability and proliferation presented approximately the same values.

The SEM analyzes of MES, at day 6, showed that PC12 cells adhere and spread in the material surface, have a rounded morphology and form clusters, with cells connected with each other. Also, the morphology of the cells was not influenced by the surface in which the cells were cultured, inner or outer surface.

In the second experiment, PC12 cells were cultured over the MES without and with microgrooves, during 14 days of culture. At day 10 of the culture, DMSO was added to differentiate the cells. In this second experience, MES with poly-L-lysine were not used because poly-L-lysine appeared to have an insignificant influence in the viability/proliferation of PC12.

Through the MTT assay, it was observed that the viability/proliferation increased during the time of culture. The MES with microgrooves was the sample that presented the smaller number of cells, although at day 14, it had a higher cell number compared to MES without microgrooves. Regarding the viability and proliferation of cells treated with DMSO, the viability/proliferation was similar to the samples not treated with DMSO.

In the SEM analyzes, at day 14 of culture, it was verified that the cells adhere and spread on the samples, present a rounded morphology and form aggregates of cells. It was also observed that cells adhere very well over the crystals of calcium carbonate, although their irregular features. The SEM images correspondent to the samples with DMSO showed that the cells began to extend neurites. However, they were small because of the few days of differentiation. With respect to the culture of cells over MES with microgrooves, it was verified that the grooves did not had the ideal width and shape to allow the adherence and orientation of the cells along them. However, the cells were able to extend small neurites along the produced microgrooves.

When compared to other studies, the MES appear to have smaller cell adhesion. In a study developed by Bechara *et al* (2010) using a scaffold of poly(ε-caprolactone) (PCL) nanowire coated with collagen and seeded with PC12 cells, cell adhesion and subsequent proliferation and viability was significantly higher on nanowire surfaces then in control surfaces. It was yet verified that proliferation and communication after 4 days of culture was higher what was indicated by the high degree of cell aggregation. Also, using SEM imaging they observed that in control surfaces (smooth surfaces) were encountered a minimal number of cells, while in nanowire PCL cells demonstrated increased spreading followed by cell colonization and communication after 4 days of culture (Bechara *et al*, 2010).

Freier and colleagues (2005) developed other experience where dorsal root ganglion (DRG) cells were cultured in tubes of chitin hydrogels and air-dried films and chitosan films. It was evaluated the adhesion and proliferation of dorsal root ganglion (DRG) neurons to the materials. The results obtained demonstrated that significantly more DRG neurons adhered to the chitosan film surface than in the other surfaces tested. Also, fewer cell clusters were formed, indicating that these cells had more affinity to the surface than for each other. It wasn't observed statistically significant differences between chitin gels and chitin films in terms of the numbers of cells bearing neurites and total neurite length. There were, however, significantly more cells bearing neurites and greater neurite lengths on chitosan films in comparison with the chitin samples (Freier *et al*, 2005)

Nervous cells are sensible to the dimensions of microgrooves as previously referred (Clark *et al*, 2001; Miller *et al*, 2001). To the alignment of the cells the microgroove width is the key parameter (Miller *et al*, 2001). Miller and coworkers (2001) demonstrated that for optimal alignment of the Schwann cells the patterns widths or spacings should be of 10 - 20 µm due to the size of Schwann cells, which varies from 5 to 10 µm. They also showed that smaller widths did not promote orientation because the microgroove widths were smaller than the cells (Miller *et al*, 2001). This is observed because microgrooves offer adequate physical and chemical guidance cues for cells to adhere and orientate their neurites (Miller *et al*, 2001).

So, the microgrooves made in the inner surface of MES should have 10 - 20 µm of width, the same utilized for alignment of Schwann cells that have the same size that PC12 cells (Farina, 2008; Hoang *et al*, 2009; Miller *et al*, 2001; Spratt, 1998). The microgrooves mechanically created in MES were smaller than expected and exhibited a V-form, so the cells were not able to grow and align within the microgrooves. However the created microgrooves had the sufficient width to allow the growth, orientation and extension of small neurites, at day 14.

In a study utilizing nonpatterned and micropatterned PLGA films were cultured PC12 cells (Yao *et al*, 2009). The microgrooves were produced with two different widths, 5 and 10 µm. They observed no apparent orientation for cells growing on PLGA films while neurites on micropatterned PLGA films showed parallel growth. Both 5 and 10 µm microgrooves guided the direction of neurite outgrowth. However, the growth of neurites on the micropatterned PLGA films with a microgroove width of 5 µm was straighter and more parallel than on the PLGA films with a microgroove width of 10 µm (Yao *et al*, 2009).

Hsu and colleagues developed an experiment with micropatterned Chitosan and PLA (Hsu *et al*, 2007). The microgrooves have feature of 20/20/3 µm (width/spacing/depth). The results showed that both Schwann cells and C6 cells were able to attach well on the two biodegradable materials and growth. The alignment of Schwann cells was slightly better on chitosan than on PLA. However, C6 cells aligned similarly well on both substrates (Hsu *et al*, 2007).

Bechara *et al* (2010) investigated the PC12 cells differentiation with NG. Through SEM images was observed that cells on nanowire PCL had a spreading morphology with individual cells interacting with each other. After 4 days was verified the initiation of neurite growth and that the neurites are interacting with the nanowire architecture. After 7 days of differentiation, significant neuronal network formation is visible, where the cells are communicating with each other by forming neurite extensions, and eventually will form a neuronal network (Bechara *et al*, 2010). In the present work, it was also observed that the cells begun to extend neurites and that they were able to interact with the MES. If the cultures were allowed to grow for more days, it would have been possible to observe the formation of a neuronal network as Bechara and coworkers reported.

Ferrari and coworkers (2010) cultured PC12 cells over nanogratings made of copolymer 2-norbornene ethylene (COC) alternatively stimulated with NGF, Forskolin, and 8-(4-chloro-phenylthio)-20-O-methyladenosine-30,50-cyclic (8CPT-2Me-cAMP) or with a combination of them. The results showed that, after NGF stimulation, PC12 cells contacting the nanograting were induced to produce two long neurites highly aligned to the underlying topography. Stimulation with Fk or co-stimulation with Fk and NGF visibly reduced neurite alignment to the nanograting. A similar result was obtained stimulating the cells with 8-Br-cAMP. Importantly, stimulation with 8-(4-chloro-phenylthio)-20-O-methyladenosine- 30, 50-cyclic (8CPT-2Me-cAMP), or co-stimulation with 8CPT-2Me-cAMP and NGF induced cells to polarize along the nanograting and neurites to align as in the control (NGF) condition. Neurites produced by PC12 cells co-stimulated with NGF and Fk or 8CPT-2Me-cAMP was visibly longer than neurites generated in control conditions. So, it was demonstrated that topographical guidance in PC12 cells is modulated by the activation of alternative neuronal differentiation pathways (Ferrari *et al*, 2010).

Thus, if the microgrooves made in the MES had the appropriate width (10 – 20 µm) and the time of culture with stimulation by DMSO was prolongated, the PC12 cells would adhere in the microgrooves and direct the neurites growth along them. In addition, the protrusions of cells could extend to others cell protrusions, allowing the communication between cells and the consequent formation of networks.

VI. Conclusion

In conclusion, MES permit the adherence and proliferation of PC12 cells, although in a lower level compared to other materials. Although the inner and outer surfaces of MES have an irregular composition, this cell line was not affected by it, been able to proliferate in the same manner in both surfaces.

The usage of microgrooves to orient the growth neuronal cells is an important feature to neuronal regeneration. PC12 cells were unable to orient their growth in the microgrooves with a width smaller than 10µm. The width that allowed this oriented growth was within 10 - 20 µm, as observed in natural microgrooves present in the MES. The small microgrooves, mechanically created, permitted the orientation of small neurites formed by PC12 in differentiation.

VII. Future perspectives

To better understand the results presented in this report it is necessary to do more studies. In future work, microgrooves with different widths, especially between 10 and 20 μm, should be tested to confirm which characteristics are adequate to promote adhesion, proliferation and aligned growth of PC12 cells along microgrooves. Also, it should be tested different needle design to produce microgrooves with a U-form.

The microgrooves produced in MES, in the experiences developed in this report, were obtained using a microhardness microscope. This proved not to be the best method to do the microgrooves and so other techniques should be investigated, as a laser.

Also, to improve the oriented growth of the axons an electric field could be applied. On the ends of the membrane, on a perpendicular direction to the microgrooves, the MES would be coated with a very thin film of gold using a sputtering technique. The gold layers would be connected to an external power supply, where the potential, intensity and frequency could be controlled, applying a low frequency electric potential. So, the growth would be oriented by the topography of the support and also by the electric stimulation provided by the potential created on the MES extremities.

Also, to allow the observation of differentiated PC12 cells, the culture period should be extended, aiming to obtain neurites that extend long protrusions to communicate with other cells forming networks oriented by the microgrooves. Another possibility would be the use of specific differentiation factors for PC12 cells, as NGF, to accelerate the differentiation and to obtain longer neurites. To confirm if the PC12 cells are differentiated, the expression of specific genes should be investigated alongside with the observation of neurites formation.

A way to confirm the results obtained in this work would be to repeat the experiences with other types of neuronal cells, as NSC, glial cell line C6 and Schwann cells, to compare the outcomes.

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