Universidade do Minho
Escola de Engenharia

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Bacterial cellulose: studies on biocompatibility, surface modification and interaction with cells

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Doutor Miguel Gama
e da
Doutora Lucília Domigues

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ABSTRACT

Bacterial Cellulose: studies on biocompatibility, surface modification and interaction with cells

A wide variety of biomaterials and bioactive molecules have been applied in tissue engineering as scaffolds in order to provide an appropriate environment to the growth and differentiation of cells. However, creating devices for biological substitutes that enhance the regeneration of neural tissues is still a challenge, because of the difficulty in providing an active stimulation of nerve regeneration. Biological scaffolds can be composed of natural polymers combined with extracellular matrix molecules and have been shown to facilitate the constructive remodeling of many tissues by the establishment of an environment necessary for the regulation of cell processes. In this context, different biomaterials have been used as scaffolds to improve interactions between material/cells and repair neurological damages. In recent years, bacterial cellulose (BC) emerged as a promising biomaterial in tissue engineering due its properties: high crystalinity, wettability, high tensile strength, pure nanofibers network, moldability *in situ* and simple production. BC has been modified to further enhance cell adhesion and biocompatibility; as an alternative to peptide chemical grafts, BC allow the use of recombinant proteins containing carbohydrates binding domains (CBMs), such as the CBM3, which has affinity by cellulose, representig a attractive way to specifically adsorb bioactive peptides on cellulose surface. The goal of this work was to modify the bacterial cellulose improving the neuronal cell affinity and producing a scaffold with potential to be used in neural tissue engineering. For this purpose, two strategies were used: 1) adhesive peptides fused to a carbohydrate binding domain with affinity to cellulose and; 2) surface modification by nitrogen plasma treatment. Also, in this work, we analized the biocompatibility in a long-term approach of two different types of BC grafts and the effect of BC nanofibers subcutaneously implanted in mice.

The recombinant proteins IKVAV-CBM3, *ex*IKVAV-CBM3 and KHIFSDDSSE-CBM3, were successfully expressed in *E. coli,* purified and stably adsorbed to the BC membranes. The *in vitro* results showed that the *ex*IKVAV-CBM3 was able to improve the adhesion of both neuronal and mesenchymal cells (MSC), while IKVAV-CBM3 and KHIFSDDSSE-CBM3 presented only a slight effect on mesenchymal cell adhesion, and no effect on the other cells. The MSCs neurotrophin expression by cells grown on BC membranes modified with the recombinant proteins was also verified. NGF was

expressed and released by cells adhered on the BC membranes, creating a microenvironment that promotes neuronal regeneration.

The nitrogen plasma treatment did not increase the wettability of the material, but increased the porosity and changed the surface chemistry, as noticed by the presence of nitrogen. XPS analysis revealed the stability of the modified material along time and autoclave sterilization. The cell adhesion and proliferation of HMEC-1 and N1E-115 cells was significantly improved in the plasma treated BC, in contrast with the 3T3 cells, revealing a cell-specific effect.

Regarding *in vivo* studies, the BC implants caused a low inflammatory reaction that decreased along time and did not elicit a foreign body reaction. A tendency for calcification, which may be related to the porosity of the BC implants, was observed. However, this tendency was different depending on the BC tested. Regarding nanofibers implants, after 2 and 4 months post implantation, mostly of injected nanofibers remained in aggregates in the subcutaneous tissue. There was infiltration of cells in these aggregates of nanofibers, mostly macrophages, and there is evidence of phagocytosis of the material by these cells. Moreover, no differences were observed between the controls and implanted animals in thymocyte populations, B lymphocyte precursors and myeloid cells in the bone marrow.

BC is a good material to be used as scaffold in tissue engineering applications. However, is still necessary to improve the interaction of cells with the material to obtain a matrix that supports the growth, differentiation and selectivity of cells. In our attempt to enhance and select neuronal attachment to BC, the recombinant proteins produced were able to improve cell adhesion and viability on BC membranes. Also, nitrogen plasma treatment proved to be an effective and economical surface treatment technique, which was also capable to improve the adhesion of endothelial and neuroblast cells to the material. Therefore, the surface modification leads to a better cell affinity with BC, probably contributing for a better biocompatibility *in vivo*. In the *in vivo* results, our work points to the necessity to further investigation to verify the tendency to BC to calcify in long-term circumstances. Meanwhile, the BC nanofibers seem to be an innocuous material in mice subcutaneous tissue, and proved to be an eligible material to production of ECM-mimetic grafts.

RESUMO

Celulose Bacteriana: estudos de biocompatibilidade, modificação de surperfície e interação com células

Actualmente, um grande número de materiais poliméricos com diferentes propriedades estão disponíveis para aplicações biomédicas. Têm sido exploradas várias abordagens com o objetivo de melhorar a interação entre os polímeros e as células, que por ser geralmente inadequada, provoca reações *in vivo* como inflamações, perdas de tecido local e encapsulamento dos implantes. Entre estas abordagens, a modificação das superfícies, como por exemplo a funcionalização dos materiais com peptídeos imobilizados ou grupos químicos incorporados, mostra vantagens na obtenção de interações específicas das células com os materiais resultando em uma melhoria na sua biocompatibilidade. A celulose bacteriana (CB) tornou-se um biomaterial em foco para aplicações biomédicas devido a sua alta resistência mecânica, hidrofilicidade, alta cristalinidade e pureza, baixo custo de produção e sua característica rede de nanofibras. Além disso, o uso de domínios de ligação à celulose é uma alternativa simples e específica de enxertar peptídeos bioativos à estrutura da celulose possibilitando uma maior afinidade celular. O objectivo deste trabalho foi modificar a CB para aumentar a afinidade de células neuronais, produzindo um *scaffold* com potencial para ser utilizado em engenharia de tecidos neuronal. Com este propósito, duas estratégias foram utilizadas: 1) o uso de peptídeos de adesão conjugados a um domínio de ligação a carbohidratos (CBM), com afinidade para a celulose e, 2) modificação da CB através do tratamento com plasma de nitrogênio. Também, dentro do âmbito deste trabalho, avaliou-se a biocompatibilidade a longo prazo da CB, tanto de implantes como de nanofibras implantados subcutaneamente em camundongos.

As proteínas recombinantes IKVAV-CBM3, *ex*IKVAV-CBM3 and KHIFSDDSSE-CBM3 foram expressas em *E.coli*, purificadas e adsorvidas de maneira estável nas membranes de CB. Os resultados *in vitro* mostraram que o *ex*IKVAV-CBM3 aumentou a adesão de células neuronais e mesenquimais, enquanto que o IKVAV-CBM3 e KHIFSDDSSE-CBM3 apresentaram apenas um pequeno efeito na adesão das células mesenquimais, e nenhum efeito nas outras células testadas. Também, a expressão de neurotrofinas pelas células mesenquimais nas membranas de CB modificadas com as proteínas recombinantes foi verificada, e verificou-se que o NGF é expresso e libertado

por estas células aderidas na CB, criando um ambiente promotor da regeneração neuronal.

O tratamento com o plasma de nitrogênio não aumentou a molhabilidade da CB, mas foi capaz de aumentar a porosidade e a química de superfície, evidenciado pela presença do grupo nitrogênio. As análises de XPS mostraram a estabilidade do material modificado 180 dias após o tratamento, e após a esterilização por autoclave. A adesão e a proliferação celular das linhagens endotelial (HMEC-1) e neuronal (N1E-115) foi aumentada significativamente na celulose tratada com plasma, em contraste com os fibroblastos 3T3, o que revelou um efeito célula-específico.

Quanto aos estudos *in vivo*, os implantes de CB causaram apenas uma reação inflamatória de baixa intensidade, que decresceu ao longo do tempo, e não estimulou reação de corpo estranho. Foi observada uma tendência para calcificar nas membranas de CB menos porosas, indicando uma relação com a porosidade dos implantes. Quanto aos implantes de nanofibras, após 2 e 4 meses de implantação, verificou-se que a maior parte das nanofibras permaneceram em agregados no tecido subcutâneo. Houve infiltração de células nesses agregados de nanofibras, sendo a maioria macrófagos, e evidências de fagocitose do material por estas células. Também, não foram encontradas diferenças entre os controles e os animais implantados nas populações de timócitos, precursores de linfócitos B e células mielóides na medula óssea.

A CB é um bom material para ser utilizado em aplicações de engenharia de tecidos. Entretanto, ainda é necessário a modificação deste material para aumentar sua interação com as células, obtendo assim uma matriz capaz de manter o crescimento, a diferenciação e a seletividade de células. Na nossa tentativa de aumentar e selecionar a adesão de células neuronais à CB, as proteínas recombinantes produzidas foram capazes de aumentar a adesão e a viabilidade celular neste material. Também, o tratamento por plasma de nitrogênio provou ser um tratamento de superfície econômico e efetivo, sendo capaz de aumentar a afinidade das células com a CB, o que poderá contribuir para um melhoramento da sua biocompatibilidade *in vivo*. Quanto aos testes *in vivo*, este trabalho aponta para a necessidade de investigação futura para verificar a tendência da CB em calcificar em circunstâncias a longo prazo. Entretanto, as nanofibras de CB parecem ser inócuas quando implantadas no tecido subcutâneo, sendo um material elegível para a produção de enxertos que mimetizem a matriz extracelular.

PUBLICATIONS

This thesis is based on the following original research or review articles:

Chapter 1: Andrade, F. K., R. A. N. Pértile, Dourado, F., Gama, F. M. P. (2010). Bacterial Cellulose: Properties, production and applications. *Cellulose, Structure and Properties, Derivatives and Industrial Uses*. A. Lejeune and T. Deprez, Nova Science Publishers, Inc.

Chapter 2: Pértile, R. A. N., Moreira, S., Andrade, F.K., Domingues, L., Gama, F. M. “Bacterial cellulose modified through recombinant carbohydrate binding module fused to bioactive peptides as a scaffold for neuronal cell culture”. Submitted

Chapter 3: Pértile, R. A. N., F. K. Andrade, Alves Jr., C., Gama, F. M. P. (2010). "Surface modification of bacterial cellulose by nitrogen-containing plasma for improved interaction with cells" *Carbohydrate Polymers* **82**(3): 692-698.

Chapter 4: Pértile, R. A. N., Moreira, S., Gil da Costa, R. M., Correia, A., Guardão, L., Gartner, F., Vilanova, M., Gama, F.M.P. Bacterial cellulose: long-term biocompatibility studies. Submitted

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ABBREVIATIONS

3D	Three dimensional
3T3	Mouse Fibroblasts cell line
ANOVA	Analysis of Variance test
ATCC	American Type Culture Collection
BASYC	Bacterial cellulose tubes
BC	Bacterial Cellulose
BC-1	Bacterial Cellulose produced by ATCC 53582
BC-2	Bacterial Cellulose produced by ATCC 10245
BCP	Plasma Treated Bacterial Cellulose
BDNF	Brain derived neurotrophic factor
BMSC	Bone marrow mesenquimal cells
cAMP	Cyclic adenosine monophosphate
CbhA	Cellobiohydrolase
CBM	Carbohydrate binding module
CBM3	Carbohydrate Binding Domain Family 3
CbpA	Cellulose binding protein A
CHO	Chinese hamster ovary cell line
CipA	Scaffolding protein
CipB	Scaffolding protein
CipC	Scaffolding protein
CNS	Central Nervous System
CNTF	Ciliary neurotrophic factor
cryoSEM	Cryo-scanning electron microscopy
Detroit 551	Human fetal skin cells
DMEM	Dulbelco´s modified Eagle medium
DNA	Deoxyribonucleic acid

DRG	Dorsal root ganglia
ECM	Extracellular Matrix
EGF	Endothelial Growth Factor
ELISA	Enzyme-linked immunosorbant assay
*ex*IKVAV	Cys-Ser-Arg-Ala-Arg-Lys-Gln-Ala-Ala-Ser-Ile-Lys-Val-Ala-Val-Ser-Ala-Asp-Arg (CSRARKQAASIKVAVSADR) (signaling domain)
FBS	Foetal Bovine Serum
FGF	Fibroblast Growth Factor
FITC	Fluorescein Isothiocyanate
GDNF	Glial-derived
GRGDY	Gly-Arg-Gly-Asp-Tys (signaling domain)
HEL	Human erythroleukemia cell line
HEMA	2-hydroxyethyl methacrylate
HMEC-1	Human Microvascular Endothelial cell line
HSC	Hematopoietic stem cells
IGF-1	Insulin-Like Growth Factor 1
IKVAV	Ile-Lys-Val-Ala-Val (signaling domain)
IMAC	Metal ion affinity chromatography
IPTG	Isopropyl-D-thiogalactopyranoside
J-111	Human histiocytic cell line
KHIFSDDSSE	Lys-His-Ile-Phe-Ser-Asp-Asp-Ser-Ser-Glu (signaling domain)
L929	Mouse Fibroblast Cells
MSC	Mesenchymal stem cell
MTS	[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]
MW	Molecular weight
N1E-115	Mouse Neuroblastoma cell line
NaOH	Sodium Hydroxyde
NCAM	Neural cell adhesion molecule

NFs	Nanofibers
NGF	Nerve growth factor
NT-3	Neurotrophin 3
NT-4	Neurotrophin 4
PBS	Phosphate Buffered Saline
PC12	Rat Pheochromocytoma cell line
PCR	Polymerase chain reaction
PEG	Poly(ethylene glycol)
PGA	Poly(glycolic acid)
PHB	Poly(3-hydroxybutyrate)
PHBV	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
PHEMA-co-MMA	Poly(2-hydroxyethylmethacrylate-co-methylmethacrylate)
PHPMA	poly[*N*-(2-hydroxypropyl)methacrylamide]
PLA	Poly(D,L lactic acid)
PLGA	Poly(D,L-lactic-co-glycolic acid)
PLL	Poly-L-lysine
PNS	Peripheral Nervous System
RGD	Arg-Gly-Asp (signaling domain)
RPMI-1640	Cell culture medium
SBB	starch-based biomaterials
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Scanning Electron Microscopy
SH-SY5Y	Human neuroblastoma cell line
SV40/Balb 3T3	BALB/3T3 cells transformed with simian virus 40 (SV40)
UPP	Unoriented polypropylene films
VEGFR-2	Vascular endothelial growth factor receptor 2
WR	Water retention values

XPS	X-Ray photoelectron spectra
YIGSR	Tyr-Ile-Gly-Ser-Arg (signaling domain)

SCOPE AND AIMS

Creating devices for biological substitutes that enhance the regeneration of neural tissues is still a challenge, because of the difficulty in providing an active stimulation of nerve regeneration. Tissue engineering uses diverse approaches to construct scaffolds that allow a good adhesion and viability of cells, and an environment which provides a regulation of cell processes. BC is a material with promising properties to be used in tissue engineering devices, and has been modified to further enhance cell adhesion and biocompatibility.

The aim of this work was to modify the BC using two different strategies: 1) the use of adhesive peptides fused to a carbohydrate binding domain with affinity to cellulose and; 2) surface modification by nitrogen plasma treatment. Also, in this work, we analized the biocompatibility in a long-term approach of two different types of BC grafts and the effect of BC nanofibers subcutaneously implanted in mice.

Chapter 1 presents a general literature review of the main subjects of this work.

Chater 2 presents the modification of BC through adsorption of recombinant proteins produced with a bioactive peptide conjugated to a carbohydrate binding domain, which has affinity by cellulose. The biological effect of the produced proteins was tested in neuronal, astrocytic and mesenchymal stem cells.

Chapter 3 presents the modification of BC membranes by nitrogen plasma treatment, their characterization, and evaluation of the biological effects of the modified BC in *in vitro* studies with endothelial, neuronal and fibroblast cells.

Chapter 4 corresponds to a long-term approach of *in vivo* studies of BC biocompatibility. In this chapter, the biocompatibility of BC implants and nanofibers implanted subcutaneously in mice are shown. This work was performed in collaboration with Immuno-Phisiology and Pharmacology Department of Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Chapter 5 summarizes the main conclusions and some future perspectives of this work.

1. General Introduction

Adapted from **Nova Publishers** (2010)

1.1 Tissue Engineering and Biomaterials

Tissue engineering is “an interdisciplinary field in which the principles of engineering and life sciences are applied toward the generation of biological substitutes aiming the creation, preservation or restoration of lost organ functions” (Langer and Vacanti 1993). For each particular clinical problem, the optimal combination of a biomaterial scaffold, cells, culture conditions and soluble regulators must be identified, allowing the regeneration of lost tissue or tissue function (Eisenbarth, Velten et al. 2007). Fig. 1.1 summarizes the tissue engineering approach for tissue regeneration.

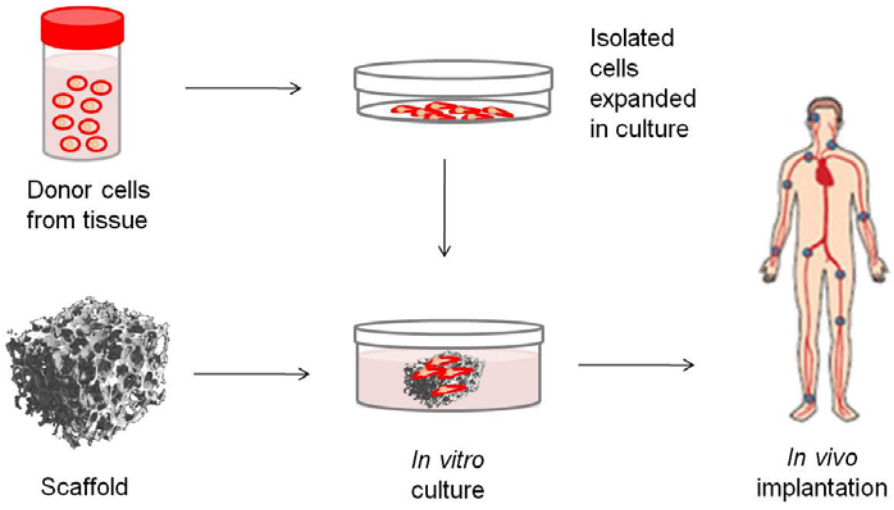

Figure 1.1 Tissue engineering approach. Cells are extracted and isolated from the donor, and expanded in culture. The scaffold is chosen depending on the tissue to be regenerated. The cells are seeded on the scaffold and implanted to regenerate the injured tissue. Adapted from: http://archive.student.bmj.com/issues/08/05/education/210.php

The challenge before tissue engineers is to provide alternatives to the dramatic lack of tissues and organs for transplantation (Mooney and Mikos 1999). Transplantation of tissues from autogeneic (from the host), allogeneic (from the same species), and xenogeneic (from a different species) sources has been a major strategy in tissue repair, but the limited availability of tissue and the issues associated with immunogenicity and disease transmission have fueled the search for a better source for tissue replacement (Chen and Mooney 2003). In this context, tissue engineering arises as a promising therapeutic solution, based on the combination of cells, biomaterials and differentiation signals (Malafaya, Silva et al. 2007).

It should be remarked that, according to some authors, the tissue engineering approach has severe limitations, namely associated to the expensive and lengthy cell growth *in vitro*. According to some authors, the regeneration of tissues and organs may be achieved alternatively, through the control of the natural regeneration mechanisms based on the recruitment of stem cells. Nevertheless, the tissue engineering tools are expected to find a growing application in the coming years, and several successful products are being developed (Okano 2004; Yang, Yamato et al. 2005).

Traditional tissue engineering methods have generally focused on one of two strategies: 1) the injection of isolated cell suspensions and 2) the use of biodegradable scaffolds supporting tissue formation (Yang, Yamato et al. 2005). However, over the years, other strategies emerged. The cell sheet engineering uses temperature-responsive culture dishes to cultivate cells that can be harvested as intact sheets simply by temperature switch. Cell sheets can be directly transplanted to host tissues or can be used to create three-dimensional structures via the layering of individual cell sheets, without the use of carrier substrates or scaffolds (Yang, Yamato et al. 2005). Another strategy consists on the use of bioactive peptides, derived from the extracellular matrix, on the site of injury. These peptides have been shown to exhibit potent chemoattrative and mitogenic activity upon endogenous progenitors and stem cells. These bioactive peptides have been used to recruit multipotential cells to the site of the injury

through *in vivo* administration. Indeed, these products seem to play a key role in the cell recruitment and constructive remodelling effect in tissue regeneration (Agrawal, Johnson et al. 2009; Reing, Zhang et al. 2009).

Thus, tissue engineering includes nowadays a variety of approaches and has driven the development of a vast variety of biomaterials with suitable properties for each envisaged application (Drury and Mooney 2003). Many of these biomaterials mimetize the composition and/or structure of the native tissues (Spector 2006), the so-called biomimetic approach, enabled by means of nano-biotechnological methods (Eisenbarth, Velten et al. 2007). According to Spector (2006), biomaterials for tissue engineering purposes should serve as a structural reinforcement of the defect, performing as a matrix for cell adhesion that facilitates or regulates cell processes such as proliferation, migration and matrix synthesis. Additionally, the biomaterial prevents infiltration of the tissue in contact with the defect, thus avoiding processes such as scarring that may impair the tissue regeneration processes (Spector 2006). Another function, also associated with the scaffolds, is to serve as carriers and delivery systems for growth factors and other biomolecular signals (Agrawal and Ray 2001). These agents stimulate biosynthetic activity and play an important role in tissue formation *in vitro* and regeneration *in vivo* (Spector 2006).

The performance and suitability of a biomaterial for a biotechnological or biomedical application is a complex function of several properties. These include the interaction with proteins and cells at the site of use; the *in vivo* degradability; the micro- and macromechanical properties; finally, the stability under sterilization conditions (Elbert and Hubbell 1996). Furthermore, specific applications imposes other specific requirements (Ratner 1996). Table 1.1 summarizes relevant characteristics of materials for specific biomedical applications.

Table 1.1 Materials properties and biomedical devices

Application	Special Property
Vascular prosthesis	Burst strength, porosity
Heart valve	Durability, hydrodynamics
Artificial heart	Flex-fatigue, resistance
Intraocular lens	Clarity, refractive index
Hip prosthesis	Lubricity
Bone cement	Quick setting, strength
Hydrocephalus shunt	Flexibility
Tendon prosthesis	Strength, flexibility

Adapted from: (Ratner 1996)

Synthetic and natural polymers have been developed as materials for the engineering of soft and hard tissues. A number of materials and scaffolds have been experimentally and/or clinically studied (Hutmacher 2001; Spector 2006). Natural hydrogel forming polymers have frequently been used in tissue engineering applications, because they are either components of or have macromolecular properties similar to the natural extracellular matrix (ECM) (Drury and Mooney 2003). In general, the scaffold should be fabricated from a highly biocompatible material, which does not have the potential to elicit an immunological, nor clinically detectable foreign body reaction (Hutmacher 2001). The criteria for material selection include the toxicology, biocompatibility, biostability or biodegradability, surface properties, scale-up, costs and other physical or chemical properties (Grosskinsky 2006). Also, control of the pore characteristics including pore volume fraction, pore diameter and orientation, which vary with host tissue type, as well as the chemical composition of the matrix, has played a critical role in the advance of the scaffolds in tissue engineering (Spector, 2006).

1.1.1 Nerve tissue engineering

The nervous system consists of two parts, the peripheral (PNS) and the central nervous systems (CNS), differing both in physiology and function (Huang and Huang 2006). Neurons and neuroglia are the cells that compose the nervous system. Neurons are the basic structural and functional elements of the nervous system and consist of a cell body (soma) and its extensions (axons and dendrites). Dendrites transmit electrical signals to the neuron cell body and the axon conducts impulses away. Glial cells, or neuroglia, are support cells that aid the function of neurons and include Schwann cells in the PNS and astrocytes and oligodendrocytes in the CNS (Schmidt and Leach 2003).

The PNS consists of the cranial nerves arising from the brain, the spinal nerves arising from the spinal cord, and sensory nerve cell bodies (dorsal root ganglia) and their processes. Peripheral nerves innervate muscle tissue, transmitting sensory input to and from the spinal column. The sensory neurons of PNS runs from stimulus receptors that inform the CNS (Fig 1.2) of the stimuli and motor neurons running from the CNS to the muscles and glands, called effectors, which take action. The CNS conducts and interprets signals as well as provides excitatory stimuli to the PNS, and is made up of spinal cord and brain, surrounded respectively by bone-skull and vertebra. Fluid and tissue also insulate the brain and spinal cord (Schmidt and Leach 2003; Huang and Huang 2006).

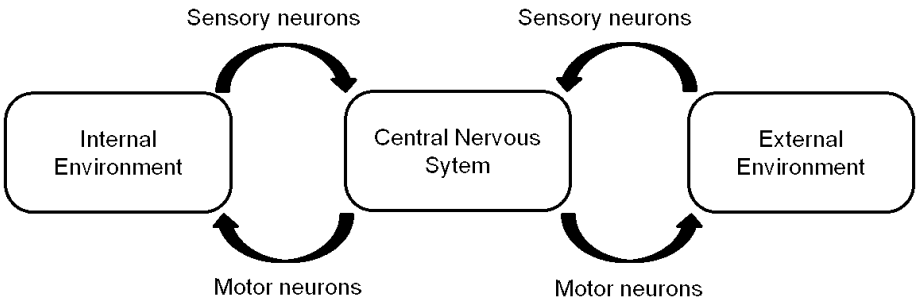

Figure 1.2 The nervous system scheme. The sensory neurons of PNS runs from stimulus receptors that inform the CNS of the stimuli and motor neurons running from the CNS to the muscles and glands. The CNS conducts and interprets signals as well as provides excitatory stimuli to the PNS. Figure adapted from Huang and Huang (2006).

In the PNS, each Schwann cell envelops a single axon and the action potentials are conducted between the myelin sheaths along the peripheral nerve fiber (Gordon and Gordon 2010). On the outer surface of this Schwann cell layer is a basement membrane similar to that found in epithelial layers. In contrast to axons in the PNS, CNS axons do not possess this continuous basement membrane and sheath of Schwann cells. Many axons are instead surrounded by an insulating myelin sheath, which is formed from dense layers of successive wrappings of the cell membrane of Schwann cells (PNS) or oligodendrocytes (CNS). Myelin serves to increase the propagation velocity of the nerve impulse, which is particularly important for those axons that extend long distances (Schmidt and Leach 2003).

The peripheral and central axonal branches of adult primary sensory neurons differ fundamentally in their response to injury: the peripheral branch regenerates after injury, but the central branch, the spinal cord, does not (Neumann, Bradke et al. 2002). The failure of the injured central branch to regenerate has multiple causes, including the presence of glial barriers and inhibitory molecules (Benfey, Bunger et al. 1985; Fawcett and Asher 1999) and the lack of some growth-promoting molecules at the injury site, which can alter the growth capacity of these neurons (Jakeman and Reier 1991; Neumann, Bradke et al. 2002). Fig 1.3 represents the CNS and PNS neurons in a situation of injury.

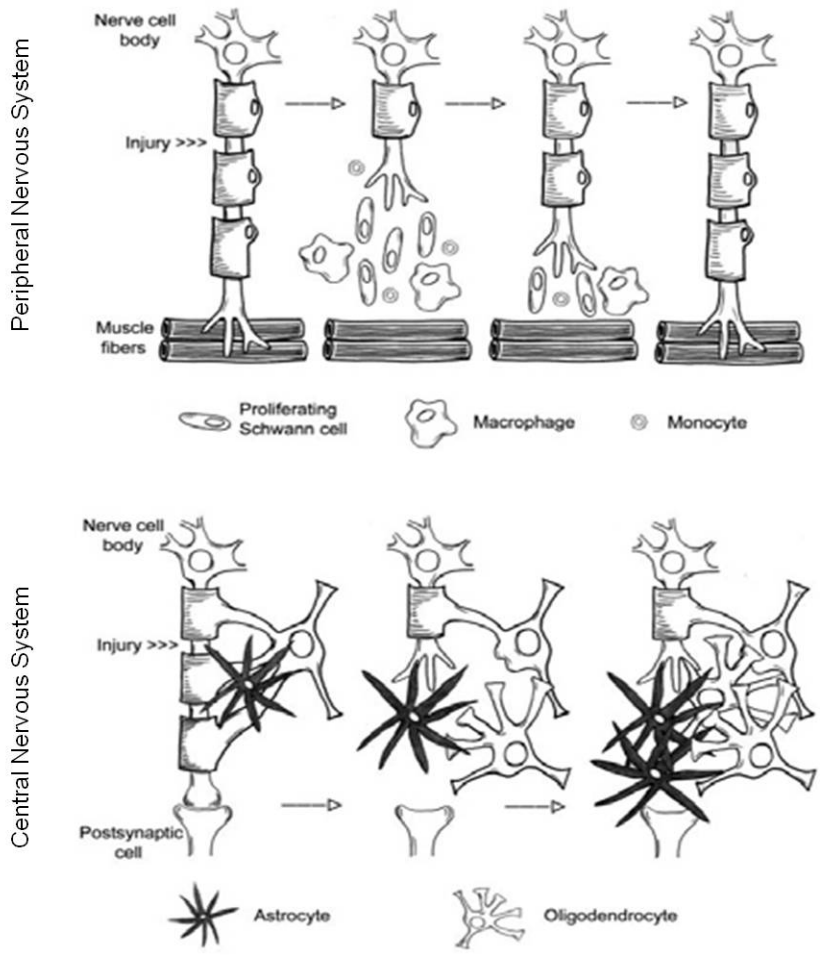

Figure 1.3 PNS axons can regenerate after an injury; CNS axons do not. Figure adapted from Bahr and Bonhoeffer (1994).

The neurodegenerative disorders of the spinal cord and brain after injury, stroke or multiple sclerosis are increasing over the years (Prabhakaran, Venugopal et al. 2008). Spinal cord injury is one of the major causes of irreversible nerve injury. A critical feature of traumatic central nervous system (CNS) damage is a cascade of secondary events that occurs after the initial injury. After a traumatic injury, there is a production of a complex inhibitory environment that poses many challenges when trying to promote regeneration (Willerth and Sakiyama-Elbert 2007). A fluid filled cavity forms at the site of injury, which becomes surrounded by a dense glial scar. Reactive astrocytes, glycosaminoglycans and other inhibitory molecules prevent neurons and other

cells from infiltrating the injury site, forming a mechanical and chemical barrier, resulting in a loss of axonal connections and a loss of motor function (Willerth and Sakiyama-Elbert 2007). These secondary events cause further tissue damage, resulting in permanent loss of function, but it can be retarded by creating a favorable microenvironment for nerve regeneration (Park, Lim et al. 2009).

The peripheral nerve lesions are common and serious injuries which generally lead to lifelong disability (Ciardelli and Chiono 2006). If there is no intervention to repair the damaged nerves, loss of function, impaired sensation and painful neuropathies will usually occur and most likely affect the patients adversely (Koh, Yong et al. 2010). The repair of peripheral nerve lesions has been attempted in many different ways, which all have in common the goal of directing the regenerating nerve fibres into the proper distal endoneurial tubes (Ciardelli and Chiono 2006). Compared to the central nervous system, peripheral axons can regenerate resulting in functional recovery, but this regenerative capacity is often incomplete and functional recovery with proximal lesions is limited. Furthermore, regeneration of axons to the appropriate targets remains a challenge with inappropriate reinnervation being an impediment to full recovery (Hoke and Brushart 2010).

Among the numerous attempts to integrate tissue-engineering concepts into strategies to repair nearly all parts of the body, neuronal repair is not satisfactory. This is partially due to the complexity of the nervous system anatomy, functioning and the inefficiency of conventional repair approaches, which were based upon single components of either biomaterials, or cells alone (Ghasemi-Mobarakeh, Prabhakaran et al. 2008). However, nerve tissue engineering is a rapidly expanding area of research providing a new and promising approach to nerve repair and regeneration (Prabhakaran, Venugopal et al. 2008). Over the recent years, knowledge of the factors influencing nerve reconstruction has increased, but still, functional outcome of peripheral nerve trauma and spinal cord injuries are often disappointing, which highlight the need to optimize therapeutical intervention. Though, the most important challenges to bioengineering research addressing nerve injuries are the physiology of the nervous system (Huang and

Huang 2006). Therefore, the challenge in nerve regeneration is to construct biological substitutes that when implanted at the lesion are capable to maintain a continuous path for regeneration, promoting the infiltration of cells to secrete inductive factors for axonal elongation, reducing scar formation (Prabhakaran, Venugopal et al. 2008). Moreover, the interaction between cells and biomaterial substrates plays an important role, especially in regulating the differentiation of cells. Despite the advances in the differentiation of stem cells to several tissue phenotypes, a biocompatible scaffold that mimics the biological and physical environment of native ECM with optimized biochemical properties, supporting the differentiation of stem cells to neuronal cells, is yet to be identified (Prabhakaran, Venugopal et al. 2008). Fig 1.4 presents the properties of an ideal neural scaffold.

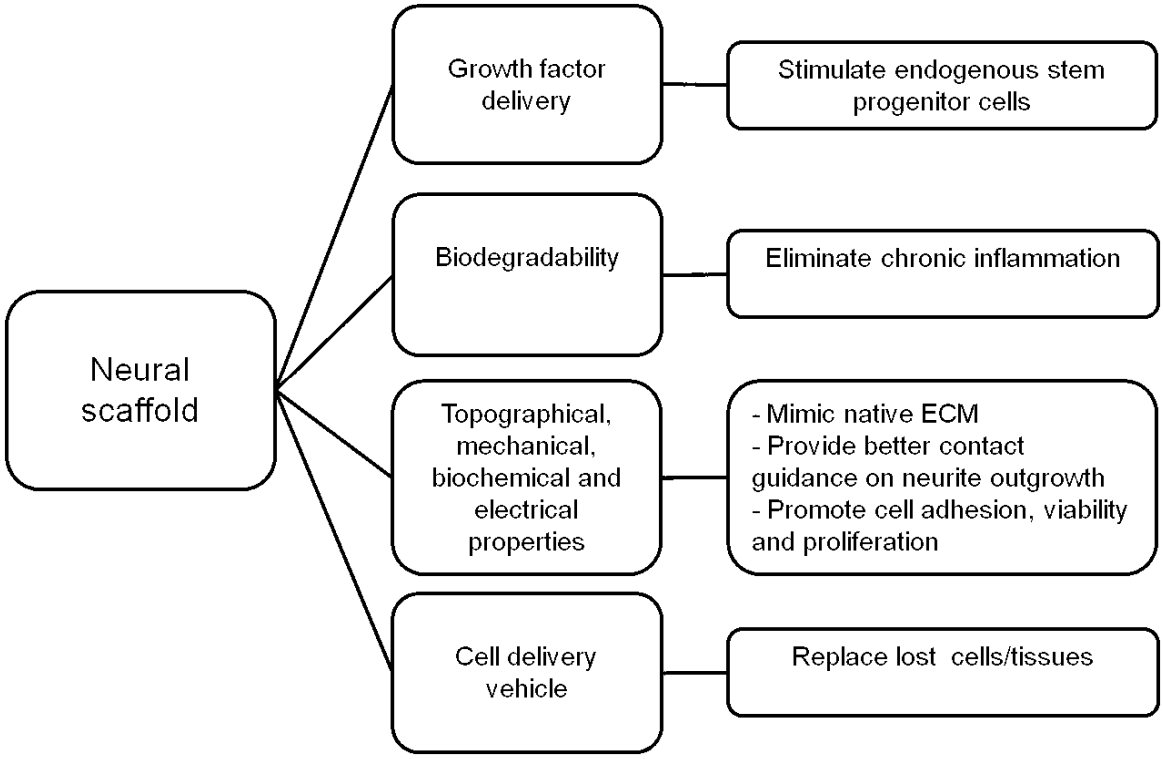

Figure 1.4 The ideal neural scaffold. Adapted from (Subramanian, Krishnan et al. 2009).

Thus, bio-engineered grafts are a promising alternative, as they can incorporate all the new developing strategies for nerve regeneration which

continually develop with the knowledge of the mechanism of regeneration (Ciardelli and Chiono 2006). A wide variety of materials have been suggested for the production of artificial devices for nerve repair, including biocompatible non-degradable and degradable materials as can be seen in table 1.2.

Table 1.2 Most studied materials for nerve regeneration and selected studies where they were used.

Material	References
Synthetic materials	
Silicon	(Smahel, Meyer et al. 1993; Zhao, Dahlin et al. 1993; Johansson, Wallman et al. 2009; He, Wang et al. 2010)
PGA: poly(glycolide)	(Nakamura, Inada et al. 2004; Fan, Gu et al. 2008; Seo, Inada et al. 2008; Huang, Cullen et al. 2009)
PLA: poly(L-lactide)	(Dendunnen, Schakenraad et al. 1993; Luciano, Zavaglia et al. 2000; Yang, Murugan et al. 2004; Sun, Kingham et al. 2010)
PHEMA-co-MMA: poly (2-hydroxyethylmethacrylate-co-methylmethacrylate)	(Dalton, Flynn et al. 2002; Midha, Munro et al. 2003; Belkas, Munro et al. 2005; Belkas, Munro et al. 2005; Katayama, Montenegro et al. 2006; Tsai, Dalton et al. 2006)
Natural materials	
Chitosan	(Wang, Ao et al. 2006; Patel, Mao et al. 2007; Fan, Gu et al. 2008; Patel, VandeVord et al. 2008; Zhang, Wang et al. 2010)
Hyaluronic acid	(Hou, Xu et al. 2005; Tian, Hou et al. 2005; Cui, Tian et al. 2006; Hou, Tian et al. 2006; Wei, Tian et al. 2007)
Collagen	(Midha, Shoichet et al. 2001; Itoh, Takakuda et al. 2002; Ahmed, Venkateshwarlu et al. 2004; Bruns, Stark et al. 2007; Bushnell, McWilliams et al. 2008; Patel, VandeVord et al. 2008; Koopmans, Hasse et al. 2009; Mollers, Heschel et al. 2009; Whitlock, Tuffaha et al. 2009)

Fibronectin	(King, Henseler et al. 2003; Phillips, King et al. 2004)
Bacterial cellulose	(Klemm, Schumann et al. 2001; Mello, Feltrin et al. 2001; Brancher and Torres 2005)
PHB: poly(3-hydroxybutyrate)	(Mohanna, Young et al. 2003; Mohanna, Terenghi et al. 2005; Bian, Wang et al. 2009)

Adapted from: (Pfister, Papaloizos et al. 2007)

Current treatment options for spinal cord injury are still limited due to the inhibitory environment created in these injuries (Willerth and Sakiyama-Elbert 2007). Several attempts for the treatment of these injuries have been described in the literature; some examples are described ahead. Novikova and colleagues, in 2008, evaluated a biodegradable tubular conduit made of poly-β-hydroxybutyrate (PHB) scaffold, predominantly with unidirectional fiber orientation, supplemented with cultured adult Schwann cells, for the axonal regeneration after cervical spinal cord injury in adult rats. After transplantation into the injured spinal cord, plain PHB conduit was well-integrated into posttraumatic cavity and regenerating axons were found mainly outside the PHB. Also, when suspension of adult Schwann cells was added to the PHB during transplantation, neurofilament-positive axons filled the conduit and became associated with the implanted cells. The results demonstrate that a PHB scaffold promotes attachment, proliferation and survival of adult Schwann cells and supports marked axonal regeneration within the graft (Novikova, Pettersson et al. 2008). Nomura and co-workers (2008) examined the implantation of extramedullary chitosan channels seeded with neural stem cells derived from rats after spinal cord transection. The survival, maturation, and functional results using neural stem cells seeded into chitosan channels, implanted between the cord stumps after complete spinal cord transection, were evaluated after 14 weeks. Channels seeded with neural stem cells showed a tissue bridge and the cells showed long-term survival. Many host axons were present in the center of the bridge in association with the transplanted cells. The channels caused minimal tissue reaction in the adjacent spinal cord. Thus, implantation of chitosan channels seeded with neural stem cells after spinal

cord transection created a tissue bridge containing many surviving transplanted cells and host axons, although there was no functional improvement (Nomura, Zahir et al. 2008). Macroporous hydrogels based on 2-hydroxyethyl methacrylate (HEMA) were used by Hejcl and colleagues (2008) to bridge a spinal cord transection in rats. Positively charged HEMA hydrogels were implanted either immediately or 1 week after spinal cord transection; control animals were left untreated. The hydrogel implants adhered well to the spinal cord tissue. After 3 months, the results showed ingrowth of connective tissue elements, blood vessels, neurofilaments, and Schwann cells into the hydrogels. The authors showed that positively charged HEMA hydrogels can bridge a posttraumatic spinal cord cavity and provide a scaffold for the ingrowth of regenerating axons. Also, the results indicate that delayed implantation can be more effective than immediate reconstructive surgery (Hejcl, Urdzikova et al. 2008). Nanofibers scaffolds for nerve guidance and drug delivery in the spinal cord were produced by Zhu and colleagues (2010). Blended polymers including poly(l-lactide acid) (PLA) and poly(lactide-co-glycotide) (PLGA) are used to electrospin nanofibrous scaffolds with a two-layer structure: aligned nanofibers in the inner layer and random nanofibers in the outer layer. Rolipram, a small molecule that can enhance cAMP (cyclic adenosine monophosphate) activity in neurons and suppress inflammatory responses, was immobilized onto the nanofibers. The nanofibrous scaffolds loaded with rolipram were used to bridge the hemisection lesion in 8-week old athymic rats. The scaffolds with rolipram increased axon growth through the scaffolds and in the lesion, promoted angiogenesis through the scaffold, and decreased the population of astrocytes and chondroitin sulfate proteoglycans in the lesion. Locomotor scale rating analysis showed that the scaffolds with rolipram significantly improved hindlimb function after 3 weeks (Zhu, Wang et al. 2010).

The current clinical gold standard for repairing peripheral nerve injuries includes end-to-end anastomosis for transected nerve that are directly adjacent, but the use of autologous nerve grafts would be required if the nerve gaps are too large and cannot be easily reconstructed by end-to-end anastomosis (Koh, Yong et al. 2010). However, because autografts result in donor-site defects and are a

limited resource, an effective substitute would be valuable (Whitlock, Tuffaha et al. 2009). The repair of the peripheral nerve gaps through tissue engineering scaffolds arises as an alternative to the use of autologous grafts to nerve regeneration. Several researchers are studying variable combinations of materials and bioactive molecules (Nisbet, Pattanawong et al. 2007; Liu 2008; Nisbet, Yu et al. 2008; Cao, Liu et al. 2009; Subramanian, Krishnan et al. 2009; Tan, Du et al. 2009; Johnson, Parker et al. 2010; Scanga, Goraltchouk et al. 2010; Suri and Schmidt 2010; Xie, MacEwan et al. 2010). In 2004, Rochkind and colleagues evaluated the efficacy of biodegradable co-polymer neurotubes containing a viscous gel with growth factors, neuroprotective agents and Schwann cells for the treatment of complete peripheral nerve injury. In 4 months, rats implanted with the composite co-polymer neurotube showed beginning of re-establishment of active foot movements. The tube was dissolved and nerve showed complete reconnection. Histological observation of the nerve showed growth of myelinated axons into the site where a nerve defect was replaced by the neurotube and into the distal part of the nerve (Rochkind, Astachov et al. 2004). In 2005, Wang and collaborators composed a scaffold with chitosan, agarose hydrogel and nerve growth factor (NGF), which was transplanted to bridge a gap of injured sciatic nerve in rat. Chitosan was used as negative control and autograft nerve as the positive one. The number and diameter of regenerating nerve fibers bridged by the scaffold performed better than the negative control and reached the level of autograft nerve group, providing a good microenvironment for nerve regeneration (Wang, Fan et al. 2005). Chen and collaborators (2006) covalently immobilized NGF, Brain derived neurotrophic factor (BDNF) and Insulin-Like Growth Factor 1 (IGF-1) on gelatin-tricalcium phosphate membrane using carbodiimide. In the *in vivo* study in rats, the membranes conduits modified with various growth factors were well tolerated by the host tissue. In the regenerated nerves, the number of axons per unit area was significantly higher in the presence of growth factors. However, the average axon size was the largest in the NGF group. In the assessment of motor and sensory recovery after nerve repair, conduits modified with various neurotrophic factors showed a more favorable outcome in compound muscle action potential (Chen, Chen et al. 2006). Wang and collaborators (2008)

developed a bilayered chitosan tube that comprises an outer layer of chitosan film and an inner layer of chitosan nonwoven nano/microfiber mesh. Also, the authors introduced glycine spacers into the CYIGSR sequence, a domain of laminin-1 that enhances Schwann cells migration and attachment, as well as neural outgrowth. The peptides were covalently bound to the nano/microfiber mesh surface of the chitosan tube so that the effects of peptide mobility on nerve regeneration could be examined. The constructed scaffolds were grafted to bridge injured sciatic nerve. These scaffolds were removed 5 and 10 weeks after implantation and results showed that the nerve regeneration into chitosan tubes, on which the CGGGGGGYIGSR peptide was immobilized, exhibited efficacy similar to that of the isograft (control), thus representing a promising candidate for promoting peripheral nerve repair (Wang, Itoh et al. 2008). Wood et al. (2010) analysed whether an affinity-based delivery system, which binds to heparin with moderate affinity and delivery NGF, affected the nerve regeneration in a rat sciatic nerve defect. After 6 weeks, histomorphometry analysis showed a higher frequency of nerve regeneration in NGF group compared to control and were similar to the nerve isograft group in measures of nerve fiber density and percent neural tissue, and larger diameter nerve fibers, suggesting more mature regenerating nerve content (Wood, Hunter et al. 2010).

Extensive attention has been devoted to develop scaffolds with inner structures mimicking the nerve-guiding basal lamina micro-channels (Hu, Huang et al. 2009). In order to maximize cell alignment and obtain a better nerve regeneration, Lietz et al. (2006) developed a resorbable, semipermeable nerve guide conduits with microstructured internal polymer filaments. To maximize Schwann cells alignment, different microtopographies were investigated. Special longitudinal microgrooves directed this cell orientation and growing axons of dorsal root ganglia. Highly oriented axon growth was observed inside nerve guide conduits of microgrooved polymer filaments. Since scar-forming fibroblasts could potentially interfere with axonal regrowth, cultures with fibroblasts, Schwann cells and dorsal root ganglia were conducted. Fibroblasts positioned on the outer nanopore containing conduit wall did not hamper neuronal and glial differentiation inside the tube (Lietz, Dreesmann et al. 2006). Valmikinathan et al. (2008)

developed a novel PLGA microsphere-based spiral scaffold designed with a nanofibrous surface to provide a good surface area, adequate mechanical properties and porosity for neuronal cell attachment and nerve regeneration. These scaffolds have an open architecture, which leaves enough volume for media influx and deeper cell penetration into the scaffolds. The *in vitro* tests conducted using Schwann cells showed that the nanofibrous spiral scaffolds promoted higher cell attachment and proliferation when compared to tubular scaffolds or nanofiber-based tubular scaffolds. Also, the surface nanofiber coating enhances the surface area, mimics the extracellular matrix and provides unidirectional alignment of cells along its direction, being a potentially scaffold to be used in nerve regeneration (Valmikinathan, Tian et al. 2008). In 2009, Hu and colleagues described a nerve-guiding scaffold composed of collagen-chitosan with inner dimensions resembling the basal lamina micro-channels of normal nerves. The scaffold has longitudinally orientated micro-channels and extensive interconnected pores between the parallel micro-channels. The efficacy of the this scaffold to bridge a long sciatic nerve defect in rats was evaluated. The results showed that the collagen-chitosan scaffold achieved *in vivo* nerve regeneration and functional recovery equivalent to an autograft, without the exogenous delivery of regenerative agents or cell transplantation (Hu, Huang et al. 2009). Also, nanofibrous conduits were used by Koh et al. (2010) in a rat sciatic nerve defect model. The conduit is made out of bilayered nanofibrous membranes with the nanofibers longitudinally aligned in the lumen and randomly oriented on the outer surface. The intra-luminal guidance channel is made out of aligned nanofibrous yarns. In addition, biomolecules such as laminin and nerve growth factor were incorporated in the nanofibrous nerve construct to determine their efficacy in *in vivo* nerve regeneration. Functional recovery was improved with use of the nerve construct (Koh, Yong et al. 2010). These findings demonstrate that scaffolds with microstructure similar to that of the nerves basal lamina has the potential for clinical usage in reconstructing peripheral nerve defects, being used as alternatives to nerve autografts for peripheral nerve regeneration.

It is a challenge to obtain successful and complete rehabilitation for peripheral nerve injuries that involve nerve transections. However, axonal

outgrowth of the peripheral nerve can be promoted if appropriate nerve repair techniques and/or nerve implant devices are used, thus reconnecting the proximal and the distal stumps for functional recovery (Koh, Yong et al. 2010). Furthermore, the existent therapies have limited capacity to reduce disease progression or damage of the CNS of adult mammals, and successful regeneration following injury or disease does not occur. However, neural tissue engineering strategies focus on developing scaffolds that artificially generate favourable cellular microenvironments to promote regeneration within the CNS, particularly in conjunction with stem cells, has generated promising results (Nisbet, Crompton et al. 2008). Table 1.3 summarizes the obstacles present in tissue regeneration of neuronal tissues, and the strategies that may be able to solve these problems.

Table 1.3 Regeneration obstacles and strategies used for neuronal tissue engineering

Peripheral nervous system	**Central nervous system**
Regeneration obstacles	
Cell body response	
Some retrograde cell death	Retrograde cell death
Ample expression of regeneration associated genes	Low expression of regeneration associated genes
Degeneration of the distal stump	Glial scar formation
Swelling of the proximal stump	Inhibitory molecules
Possible gap between nerve stumps	Myelin-associated glycoprotein
	Chondroitin sulfate proteoglycans
Strategies for repair	
Guidance therapies	
Autologous tissue grafts	Peripheral nerve and embryonic spinal cord grafts

Acellular tissue grafts	Support matrices
Nerve conduits	
Biomolecular therapies	
Neurotrophic factors	Neurotrophic factors
Regeneration-associated genes	Regeneration-associated genes
Antiapoptosis genes	Antiapoptosis genes
	Blocking inhibitory biomolecules
Cellular therapies	
Schwann cells	Schwann cells
Macrophages	Macrophages
Stem cells	Stem cells
Genetically modified cells	Genetically modified cells

Adapted from: (Schmidt and Leach 2003)

1.2 Stem cells and Tissue Engineering

Controlling microenvironments in damaged tissues is a challenging problem in regenerative medicine and tissue engineering, where the modulation of the microenvironment may allow the control over the regenerative processes. Depending upon the type of tissue injured, various bioactive molecules, specific cells, peptides, and scaffolds have been used for this purpose (Park, Lim et al. 2009). The use of stem cells in tissue engineering constructs is a promising strategy, because these cells can express a variety of growth factors important for tissue regeneration and cell differentiation.

The stem cells can be defined by two distinct traits: self-renewal, which is the process where a single cell gives rise to two cells, and differentiation ability where a progenitor cell differentiate to a mature cell type upon specific cues and signals (Barzilay, Levy et al. 2006). There are different sources of stem cells in

different tissues. The stem cell population is comprised of two main cell types: embryonic stem cells and adult stem cells. The adult stem cells have the capacity to differentiate along their lineage of origin, but also, there have been reports of the ability of these cells to differentiate along different lineages than its original organ, showing multipotency (Barzilay, Levy et al. 2006). Bone marrow provides continuous source of stem cells: the hematopoietic stem cells (HSCs) and nonhematopoietic or mesenchymal stem cells. The stem-like cells from nonhematopoietic tissues are currently referred as mesenchymal stem cells (MSCs), because of their ability to differentiate into cells that can roughly be defined as mesenchymal or marrow stromal cells, and they appear to arise from the complex array of supporting structures found in marrow (Prockop 1997). MSCs adhere strongly to tissue culture plastic and are capable of multipotent differentiation into osteoblasts, chondroblasts, adipocytes and myoblasts. Some studies also indicate that bone marrow MSCs can be induced to differentiate to neuron-like cells (Sanchez-Ramos, Song et al. 2000; Woodbury, Schwarz et al. 2000; Black and Woodbury 2001). Moreover, there is evidence for MSC differentiation into functional glial cells, mainly to astrocyte and oligodendrocyte phenotypes (Suzuki, Taguchi et al. 2004; Blondheim, Levy et al. 2006). Expanded, plastic adherent MSCs often are positive for the surface markers CD73, CD90 and CD105, but negative for CD11b, CD19, CD34 and CD45 (Montzka, Lassonczyk et al. 2009) and represent a minor fraction of the total nucleated cell population in marrow, having a fibroblastic morphology in culture (Barry and Murphy 2004).

Although the mechanism underlying the stem cells beneficial effect in the treatment of diseases is not elucidated, its potential has been demonstrated using different approaches. Cell replacement is one of those, consisting in the direct replacement of the degenerated cells by functional cells. Also, the transplantation of stem cells can provide support to affected cells by secreting cytokines and neurotrophic factors, which means the creation of a neuroprotective environment. Another approach is the gene delivery, using stem cells as vehicles to deliver specific supportive genes to the affected area (Barzilay, Levy et al. 2006). MSCs have been exploited in the treatment of neurological diseases. Since the survival

and migration of human MSCs grafted into rat brains was demonstrated, the possibility that such cells might act as suitable tools for promoting CNS repair has been raised (Montzka, Lassonczyk et al. 2009). MSC administration has been shown to promote neuronal survival and limit the severity of neurological impairment in animal models of induced stroke and traumatic brain injury, as well as promote recovery of motor function in mice (Chen, Chai et al. 2001; Li, Chen et al. 2001; Lu, Mahmood et al. 2001; Zhao, Duan et al. 2002). Direct implantation of MSCs into the spinal column has also been shown to promote functional recovery following a standardized contusion injury (Chopp, Zhang et al. 2000; Hofstetter, Schwarz et al. 2002; Crigler, Robey et al. 2006). Although the neuroprotective effects of MSCs may result from their ability to replace the diseased or damaged neurons via cellular differentiation, it has been suggested that the effects could also be credited to MSCs' ability to produce important factors (neurotrophic factors) that support neuronal cell survival and promote nerve fiber regeneration at the sites of injury (Abe 2000; Li, Chen et al. 2002; Mahmood, Lu et al. 2004; Jiang, Lv et al. 2010).

Some of the most common growth factors used to promote neural tissue engineering are neurotrophins (Willerth and Sakiyama-Elbert 2007). Neurotrophins are a family of proteins that induce the survival, development and function of neurons (Coumans, Lin et al. 2001). The family includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) also known as just NT-4 and NT-5, and are derived from a common ancestral gene, are similar in sequence and structure, and are therefore collectively named neurotrophins (Hallbook 1999). Outside of the neurotrophin family, other factors of importance are ciliary neurotrophic factor (CNTF), glial cell line-derived growth factor (GDNF), and acidic and basic fibroblast growth factor (FGFs) (Schmidt and Leach 2003). Table 1.4 summarizes the type of neuronal response related to neurotrophins.

Table 1.4 Neuron response to neurotrophins

Neural response promoted	Neurotrophic factors
Motor neuron survival and outgrowth	BDNF, NT-3, NT-4/5, CNTF, GDNF
Sensory neuron survival / outgrowth	NGF, NT-4/5, GDNF / NGF, BDNF, NT-3
Spinal cord regeneration	NGF, NT-3, CNTF, FGFs
Peripheral nerve regeneration	NGF, NT-3, NT-4/5, CNTF, GDNF, FGFs
Sensory nerve growth across the PNS-CNS transition zone	NGF, NT-3, GDNF, FGFs

Adapted from: (Huang and Huang 2006)

The administration of neurotrophins is a potential approach to the therapy of neurodegenerative disorders or injuries, spinal cord and brain trauma, but limitations regarding its effective delivery and potential side effects have limited the clinical application of this strategy (Jiang, Lv et al. 2010). The influence of the neurotrophins in nerve regeneration has been the focus of extensive research (Anand, Birch et al. 1994; Houweling, Bar et al. 1998; Houweling, Lankhorst et al. 1998; Terenghi 1999; Blesch 2000; Jones, Oudega et al. 2001; Yin, Kemp et al. 2001; Blesch, Lu et al. 2002; Sahenk, Nagaraja et al. 2003; Tuszynski, Blesch et al. 2003; Serpe, Byram et al. 2005; Vogelin, Baker et al. 2006; Kwon, Liu et al. 2007; Li, Li et al. 2008; Chu, Li et al. 2009; Guzen, Leme et al. 2009; Xu, Chen et al. 2009). The administration of BDNF or NT-3 in hemisection and spinal cord transplant in the adult rat was showed to increase the axonal growth within the transplant and prevent the atrophy of axotomized supraspinal neurons (Bregman, McAtee et al. 1997; Bregman, Broude et al. 1998). In addition, neurotrophins can increase the expression of regeneration-associated genes within the cell bodies of the injured axons (Broude, McAtee et al. 1999; Coumans, Lin et al. 2001). The effect of intramedullary infusion of BDNF, NGF, or NT-3 on the regeneration after spinal cord injury in adult rats was tested by Namiki and colleagues (2000). Invasion and proliferation of Schwann cells and formation of peripheral myelin were more prominent at the injury site in the BDNF-treated animals indicating that

continuous intramedullary infusion of BDNF provides neuroprotection and enhances some regenerative activity after spinal cord injury (Namiki, Kojima et al. 2000). Bloch and colleagues (2001) studied the continuously release of neurotrophins by synthetic nerve guidance channels in the transected rat dorsal root. Four weeks after the induced lesion, the BDNF showed a limited effect on axonal regeneration, but NGF and NT-3 powerfully promoted regeneration of myelinated axons. NGF had a potent effect on the regeneration of unmyelinated axons. This study suggests that the slowly and continuously releasing of the neurotrophins NGF and NT-3 can overcome the limited regeneration of transected dorsal root (Bloch, Fine et al. 2001). Yu and colleagues produced NGF-containing polymeric microspheres (PLGA-PLA) and mixed with fibrin glue to develop nerve grafts for prolonged, site-specific delivery of NGF. To assess nerve regeneration the authors used a model of sciatic nerve gaps in rats. Sixteen weeks after nerve repair, the ratio of conserved muscle-mass was lower in the NGF-treated group than in the autograft group. Image analysis revealed that axonal diameter, axon number, and myelin thickness was similar to NGF-treated acellular grafting and autografting, showing that this method of sustained site-specific delivery of NGF can enhance peripheral nerve regeneration across short nerve gaps repaired with acellular nerve grafts (Yu, Peng et al. 2009).

It has recently been demonstrated that MSCs, even without any induction, are able to secret neurotrophins such as NGF, BDNF, GDNF, CNTF and NT3, thus providing a natural source for neurotrophins (Jiang, Lv et al. 2010) that can be used in tissue engineering constructs. Tohill and colleagues (2004) exposed bone marrow mesenchymal stem cells (BMSCs) to glial growth factor and transplanted into nerve conduits in the rat sciatic nerve. MSCs maintained glial markers expression and enhanced nerve regeneration, with significant Schwan cell regeneration (Tohill, Mantovani et al. 2004). Rat amniotic fluid MSCs were used by Pan and colleagues (2007), embedded in fibrin glue, to be delivered to the injured nerve. High levels of expression of BDNF, GDNF, CNTF, NGF and NT-3 were demonstrated in these MSCs. Also, motor function recovery, the compound muscle action potential, and nerve conduction latency showed significant improvement in rats treated with these cells. The results revealed less

fibrosis and a high level of expression glial markers at the injury site. The authors hypothesize that the increased nerve regeneration verified is due to the neurotrophic factors secreted by the MSCs (Pan, Cheng et al. 2007). Chen and collaborators (2007) also tested the beneficial effects of implanted BMSCs on sciatic nerve regeneration; when compared to silicon tubes control, animals subjected to this treatment improved walking behaviour, reduced loss of muscle weight and greater number of regenerating axons within the tube was verified. The authors associated the regenerative potential of BMSCs with the neurotrophic factors produced by these cells, leading to a promoting effect on nerve regeneration (Chen, Ou et al. 2007). Wang and colleagues used MSCs to promote peripheral nerve regeneration in a rat sciatic nerve gap model. The influence of MSCs on the proliferation of Schwann cells and on the neurotrophic factor expression in nerve regeneration was evaluated. The results confirmed that administration of MSCs into nerve conduits stimulated Schwann cells proliferation and axonal outgrowth, and also up-regulated expression of nerve skeleton molecules, neurotrophic factors and their receptors within the rat regenerating nerves (Wang, Ding et al. 2009). Zheng and Cui (2010) developed and tested chitosan conduit to use in peripheral nerve reconstruction combined with BMSCs. The BMSCs transplanted can differentiate into neural stem cells *in vivo*, and the chitosan combined with BMSCs showed to bridge neural gap better resulting from the differentiation effects of the BMSCs (Zheng and Cui 2010). More examples of the use of stem cells for neuronal tissue engineering are reported in several studies (Cuevas, Carceller et al. 2002; Lu, Jones et al. 2003; Cuevas, Carceller et al. 2004; Caddick, Wiberg et al. 2005; Crigler, Robey et al. 2006; Keilhoff, Stang et al. 2006; Pan, Yang et al. 2006; Kwon, Song et al. 2009; Makar, Bever et al. 2009; Pan, Chen et al. 2009; Rooney, McMahon et al. 2009; Sadan, Shemesh et al. 2009; Shi, Zhou et al. 2009; Wilkins, Kemp et al. 2009; Yagihashi, Mizukami et al. 2009). Furthermore, MSCs produce other neuroregulatory molecules in addition to neurotrophins that play a role in neuronal cell survival and neuritogenesis, and accounts for the ability of these cells to engraft, migrate and affect repair within nervous system (Crigler, Robey et al. 2006). These cells are

an important tool to neuronal tissue engineering and seem to be a promising treatment for neurological diseases and injuries.

1.3 Bacterial Cellulose as a biomaterial for tissue engineering

Bacterial cellulose (BC) is a biomaterial produced by bacterial strains from the genera *Acetobacter*, *Agrobacterium*, *Pseudomonas*, *Rhizobium* and *Sarcina*, the last one being the only genus of Gram-positive bacteria in this field (Jonas and Farah 1998). Interestingly, only a few bacterial species, taxonomically related to this genus, extracellularly secrete the synthesized cellulose as fibers. Figure 1.5 shows an image of bacterial cellulose network and the bacterial cells published by Klemm et al. (2001).

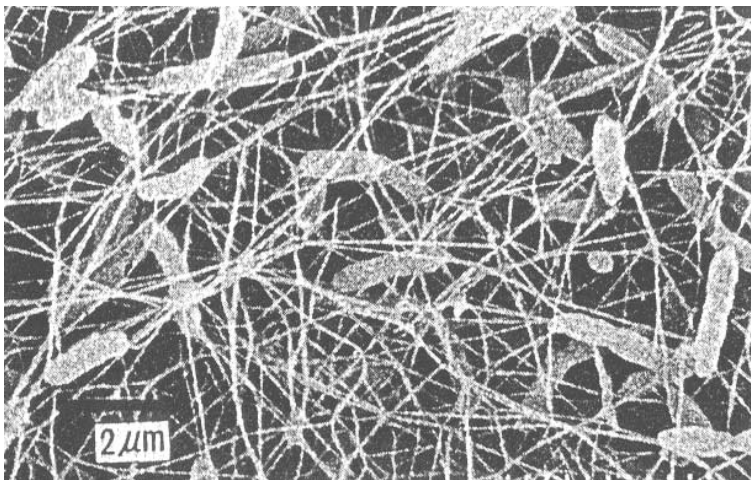

Figure 1.5 Bacterial cellulose and bacterial cells. Image from Klemm, et al. (2001).

Special attention was given to strains from *Gluconacetobacter xylinus* (=*Acetobacter xylinum*), first described by Brown in 1886 (Brown 1886). While the secreted cellulose is identical to the one produced by plants, regarding the molecular structure, it is chemically pure, *i.e.* not mixed with non-cellulosic polysaccharides (Jonas and Farah 1998; Vandamme, De Baets et al. 1998; Klemm, Schumann et al. 2001; Amano, Ito et al. 2005; Helenius, Backdahl et al.

2006). Its unique properties account for its extraordinary physico-chemical and mechanical behaviour, resulting in characteristics that are quite promising for modern medicine and biomedical research (Watanabe, Eto et al. 1993; Iguchi, Yamanaka et al. 2000; Klemm, Schumann et al. 2001; Svensson, Harrah et al. 2004; Czaja, Krystynowicz et al. 2006; Czaja, Young et al. 2007).

1.3.1 Biosynthesis, Structure and Properties

The classical medium to culture *G. xylinus* and maximize the growth and cellulose production was described by Hestrin and Schramm. The pH of the medium is 6 and the optimum growth temperature is 30 ºC, though the bacteria grow well over a temperature range of 25 to 30 ºC. The static culture leads to the production of a cellulose pellicle holding bacterial cells floating on the surface medium. In a culture medium aerated by shaking, bacteria grow faster, but less cellulose, presented as ball-shaped particles, is produced. When *G. xylinus* is cultured on solid medium, the colonies have a dry, wrinkled appearance (Hestrin and Schramm 1954; Cannon and Anderson 1991).

The ultrastructure of the cellulose synthesis apparatus is best understood in *G. xylinus.* The cellulose synthase is considered the most important enzyme in the bacterial cellulose biosynthesis. The cellulose synthase operon codes protein complexes aligned along the long axis of the cell. Cellulose synthesizing complexes are present in the surface of the bacteria, next to the cell membrane pores where the cellulose fibrils are extruded through, associating with other fibrils and making up the ribbon of crystalline cellulose (Jonas and Farah 1998; Amano, Ito et al. 2005). Each bacterium synthesizes a cellulosic ribbon with a width ranging from 40 to 60 nm, parallel to the longitudinal axis of the bacterial cell. The ribbon of cellulose is composed of microfibrils with around 1.5 nm thickness, secreted through extrusion sites in the outer membrane of the bacterium. Then, the microfibrils aggregate into 3 to 4 nm microfibrils via crystallization of adjacent glucan chains and finally, together, form the larger cellulosic ribbon (Cannon and Anderson 1991).

Several studies were developed to clarify the physiologic role of cellulose. As the cellulose matrix is less dense than water, it has been proposed to allow maintaining the bacterial cells in an oxygen-rich environment. Additionally, it allows protecting the bacteria from ultraviolet light, competing microorganisms and heavy-metal ions, while retaining the moisture and allowing nutrient supply by diffusion (Hestrin and Schramm 1954; Ross, Mayer et al. 1991; Iguchi, Yamanaka et al. 2000; Klemm, Schumann et al. 2001).

As *Gluconacetobacter* microorganisms are mandatory aerobes, under static conditions, BC is synthesized at the air/liquid interface of the culture medium (Jonas and Farah 1998; Klemm, Schumann et al. 2001). Other relevant aspects for the BC production are the carbon and nitrogen sources and concentration, the pH and temperature, and the surface area of the fermentation system. All these aspects affect the cellulose production as well as the membrane properties, in static or agitated cell culture. Also, differences in the bacterial strains play an important role in the microstructure and production rate. Figure 1.6 shows a membrane produced by ATCC 10245 *G. xylinus* strain (Kouda, Yano et al. 1997; Jonas and Farah 1998; Hwang, Yang et al. 1999; Ramana, Tomar et al. 2000; Klemm, Schumann et al. 2001; Krystynowicz, Czaja et al. 2002; Bodin, Backdahl et al. 2007).

Figure 1.6 Bacterial cellulose pellicle produced by *G. xylinus* in static culture (ATCC 10245).

Besides macroscopic morphological differences, BC produced in static and agitated cultures differs also at various structural levels. While the fibril network remains the same, there are some differences in the structure of crystals and molecular chains. The crystallinity and cellulose I alpha content, as well as the degree of polymerization, is lower in agitated than in static culture (Valla, Ertesvåg et al. 2009).

As referred above, the bacterial and vegetable celluloses have the same molecular structure, both being built up of β(1→4)–linked D-glucose units. The degree of polymerization is however rather different, about 13000-14000 for plants and 2000-6000 for bacterial cellulose. Both celluloses are highly crystalline; differing in the arrangement of glucosyl units within the unit cells of the crystallites, and several studies suggests that these celluloses are synthesized by enzymatic complexes that differ at the molecular level. Also, this bacterial polysaccharide is secreted free of lignin, pectin, hemicelluloses and other biogenic compounds, which are associated with plant cellulose (Jonas and Farah 1998; Brown and Saxena 2000; Klemm, Schumann et al. 2001).

Morphology - The gelatinous BC membrane formed in static culture is characterized by a 3D ultrafine fibrous network structure, containing about 99% water. The randomly assembled ribbon-shaped fibrils are less than 100 nm wide and composed of elementary nanofibrils, aggregated in bundles with lateral size of 7-8 nm. The crystallinity degree of BC is in the range of 60-90% (Yamanaka, Watanabe et al. 1989; Klemm, Heublein et al. 2005; Nakagaito, Iwamoto et al. 2005; Backdahl, Helenius et al. 2006; Bodin, Ahrenstedt et al. 2007). Crystallographically, BC is a Cellulose I, with 60% Iα /40% I*β* (Iguchi, Yamanaka et al. 2000; Bodin, Ahrenstedt et al. 2007). The crystallographic molecular arrangement may influence the physical properties, as the allomorphs have different crystal packing, molecular conformation, and hydrogen bonding (Klemm, Heublein et al. 2005; Bodin, Ahrenstedt et al. 2007). In 2006, Sanchavanakit characterized BC pellicles obtained after 48 hours culture: the surface area of the air-dried BC films was 12.6 m^2/g, with a pore size distribution ranging from 45 to 600 Å. The pore diameter of the air-dried film was inferior to 0,1 μm; however,

when the air-dried pellicle was swollen with water, at 30 ^{o}C, the apparent pore diameter raised to 0.2-1.0 μm (Sanchavanakit, Sangrungraungroj et al. 2006). Due to its high crystallinity and small fiber diameter, BC possess excellent mechanical strength and high surface area when compared to plant derived cellulose (Sokolnicki, Fisher et al. 2006) and the application and biological function of celluloses are based on its distinct fiber morphology (Klemm, Heublein et al. 2005). Figure 1.7 show a BC membrane with mammalian cells adhered on the surface, and a detail of BC membrane surface.

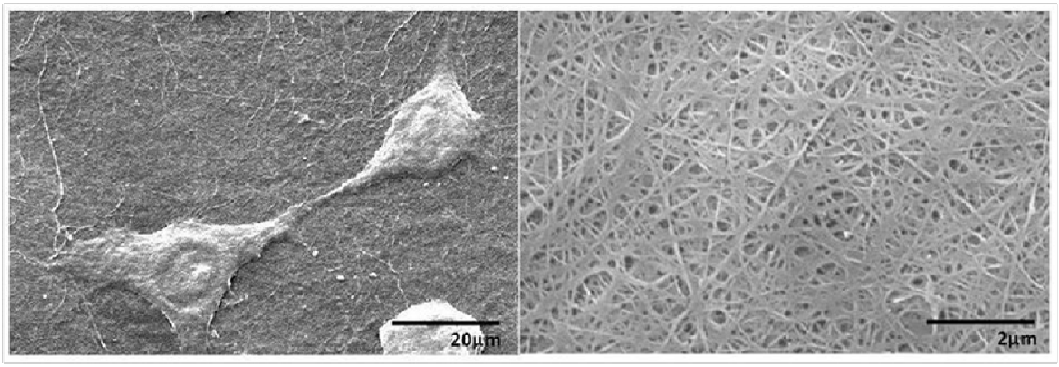

Figure 1.7 Scanning eletron microscopy of bacterial cellulose. (A) Fibroblasts adhered on bacterial cellulose membranes after 24h in culture; (B) detail of BC membranes surface.

Mechanical properties **–** Both the micro and macrostructure of BC are influenced by the growing culture environment and the treatment after synthesis. According to Iguchi, a BC pellicle obtained after 7 days of culture and air-dried at 20 ^{o}C and low pressure, presents a Young's modulus of 16,9 GPa, tensile strength of 256 MPa and elongation of 1,7% (Iguchi, Yamanaka et al. 2000). However, when a pellicle was dried through the heat-press method described by Iguchi (Iguchi, Mitsuhashi et al. 1988) and an excess of pressure (490 – 1960 kPa) was applied, the tensile strength as well as elongation tend to decrease, while the Young modulus remains constant. According to Sanchavanakit (2006), a BC dried film (from a 48h grown culture) with a thickness of 0.12 mm presents a tensile strength and break strain of 5.21 MPa and 3.75%, whereas for the wet films the values are 1.56 MPa and 8.00%, respectively (Sanchavanakit, Sangrungraungroj et al. 2006). The high Young's modulus and tensile strength of

BC films seems to result from its high crystallinity, high planar orientation of ribbons pressed into a sheet, ultrafine structure, and the complex network of the ribbons (Nishi, Uryu et al. 1990).

Water holding capacity - BC is highly hydrophilic, holding over 100 times its weight in water. Klemm and colleagues showed that the "never dried" BC has water retention values (WR) in the range of 1000%, drastically decreasing after air-drying to values that can be compared with those of plant cellulose, 106% and 60%, respectively. The method of drying has been shown to affect the BC porosity, freeze-drying (WR of 629%) being reported as the most effective method to preserve the porous structure (Klemm, Schumann et al. 2001).

Permeability – Sokolnicki *et al.* carried out mass transfer experiments to characterize the transport of biomolecules (namely vitamin B12, lysozyme and bovine serum albumin, with molecular weight of 1355 Da, 14.3 kDa and 66.3 kDa, respectively) through hydrated BC membranes. The results indicated a dual transport mechanism of the solute through the continuous water phase and cellulose matrix, with some hindrance of molecular diffusion via fiber obstruction. Also, the 94% membrane porosity and its morphology indicated the existence of micro-channels of varying size, through which solute diffusion occurs. The diffusivities of all tested solutes could be attributed primarily to hydrodynamic and entropic exclusion and only slightly to partitioning and adsorption in the case of low molecular weight molecules (Sokolnicki, Fisher et al. 2006).

1.3.2 Medical Applications

The biocompatible nature of cellulose-based materials, such as oxidized cellulose, regenerated cellulose hydrogels, sponge cellulose and bacterial cellulose, has allowed comprehensive research targeted at medical applications (Martson, Viljanto et al. 1998; Fricain, Granja et al. 2002; Entcheva, Bien et al. 2004; Muller, Muller et al. 2006; Shi, Chen et al. 2009). Representative examples BC-based scaffolds for tissue engineering include vascular grafts, cartilage, neural regeneration and wound dressings.

The interaction between cells and BC has been investigated by several research groups. In 1993, BC was described as a substrate for mammalian cell culture by Watanabe and colleagues (Watanabe, Eto et al. 1993). Adhesion to BC was observed using anchorage-dependent cell lines (L929 mouse fibroblasts, Detroit 551, HEL, mouse 3T3 Swiss, SV40/Balb 3T3, CHO, Human J-111 and Human epidermal Keratinocytes). Modification of the BC surface, to improve the interaction with cells, involved the introduction of electrical charge and adhesive proteins, such as collagen type I, collagen type IV, fibrin, fibronectin or laminin (Watanabe, Eto et al. 1993). Andrade *et al*. improved the adhesion of fibroblasts on BC pellicles modified using four recombinant proteins containing cellulose-binding module and an adhesion peptide (Andrade, Moreira et al. 2008).

The interaction of BC films with human transformed skin keratinocytes and human normal skin fibroblasts was evaluated (Sanchavanakit, Sangrungraungroj et al. 2006). The results demonstrated that BC supports the proliferation of both cell types, with no signs of toxicity; the keratinocytes exhibited normal cell proliferation, spreading and also maintained the normal phenotype, while for the fibroblast culture the pattern of cell distribution and stability on BC film was poorer. Moreover, the migration of keratinocytes on a BC film was comparable to that of a polystyrene plate. Pértile and colleagues, in 2007, found a similar behavior when studying the interaction between BC pellicles and skin fibroblasts (Pértile, Siqueira et al. 2007).

In an *in vivo* biocompatibility study, BC was subcutaneously implanted in mice, for a period of up to 12 weeks (Helenius, Backdahl et al. 2006). BC was shown to integrate well into the host tissue, with cells infiltrating the BC network and no signs of chronic inflammatory reaction or capsule formation. The formation of new blood vessels around and inside the implants was also observed, evidencing the good biocompatibility of the biomaterial.

1.3.3 BC in tissue regeneration

The BC is used in wound dressing for a long time. Since 1990, BC has been tested as a temporary dressing and skin healing. The advantage of BC in wound healing is that these membranes do not require daily exchange, normally mandatory with other wound dressings and create a protective, hypoxic, moist environment optimizing the skin healing (Wouk, Diniz et al. 1998; Osman, Souza et al. 2007). BC also was tested as dural substitute (Mello, Feltrin et al. 1997), chronic venous insufficiency and lower-leg ulceration (Alvarez, Patel et al. 2004), and repair of chronic lower extremity ulcers (Portal, Clark et al. 2009). BC membranes are already used in dental implants, periondontal disease treatment and guided bone regeneration - alone or in association with osteointegrated implants - proving a good alternative for guided tissue regeneration (Novaes and Novaes 1993; Novaes, Novaes et al. 1993; Novaes and Novaes 1995; Novaes and Novaes 1997; dos Anjos, Novaes et al. 1998).

The mechanical properties closely related to native cartilage and superior to other materials makes the BC a good material for cartilage tissue repair (Svensson, Harrah et al. 2004). BC scaffolds showed to support growth of chondrocytes, allowing cell migration and ingrowth in vitro, and good integration with the host tissue when implanted (Bodin, Concaro et al. 2007; Oliveira, Souza et al. 2007).

In 2006, BC was considered as a novel biomaterial for tissue engineered blood vessels (Backdahl, Helenius et al. 2006), with its good mechanical properties, interaction with smooth muscle cells and good *in situ* tissue regeneration. Furthermore, stents coated with BC had an accelerated re-endotelialization of the area covered by the stent, acting as a barrier to the migration of muscle cells, thus representing a promising strategy for the prevention and treatment of restenosis in endovascular procedures (Negrão, Bueno et al. 2006). Other authors also tested BC in vascular grafts, with promising results (Klemm, Schumann et al. 2001; Putra, Kakugo et al. 2008; Wippermann, Schumann et al. 2009).

1.3.1.1 BC in nerve regeneration

Klemm and colleagues tested the micronerve reconstruction of rat sciatic nerve using bacterial cellulose tubes (BASYC®) (Klemm, Schumann et al. 2001). The regeneration of the functional nerve, following 10 weeks of surgery, was improved as compared to the uncovered anastomosed nerve. The reappearance of acetylcholine as the transmitter of nerve impulses to the executive organ was observed. In the same animal model, the BASYC® was used as a drug depot of neuroregenerative substances, allowing an earlier return of innervation and the functional recreation of the paralyzed legs, as evaluated by the walking behaviour scores. Mello and co-workers, in 2001, used bacterial cellulose sheets to envelop peripheral nerve lesions with loss of neural substance, in dogs, and analyzed the degree of inflammatory reaction and axon realignment in the sciatic nerve (Mello, Feltrin et al. 2001). A moderate fibrous reaction caused by the BC sheets implanted in the peripheral nerve, and also realignment and axonal growth through the injury were observed. Brancher and Torres observed rats' facial nerves repair following trans-section (Brancher and Torres 2005). The extremities were approximated with a plain epineural suture stitch and surrounded with BioFill® sheets. The researchers found that the BC sheets improved guidance of the nerve fibers, allowing the concentration of neurotrophic factors, which consequently promoted the nerve regeneration.

1.4 Bacterial cellulose modification: Improving the BC Properties for Biomedical Applications

Biocompatibility is one of the main requirements for any biomedical material. It can be defined as the ability to remain in contact with living tissue without causing any toxic or allergic side effects, simultaneously performing its function (Czaja, Young et al. 2007). Almost all biological interactions are mediated by specific biorecognition, like the high-affinity binding of receptors on cell surfaces to

ligands on biomaterial surfaces (Elbert and Hubbell 1996). Based on the understanding of the dominance of the biorecognition process on cell behaviors, two main strategies in surface engineering of biomaterials are often employed. Firstly, the material surface properties are modulated to a state that the adsorbed proteins can maintain their normal bioactivities. This method, however, cannot induce specific cell behaviors due to the nonspecific protein absorption. The second strategy is to directly immobilize certain biomolecules on the biomaterial surfaces to induce specific cellular responding (Ma, Mao et al. 2007). Also, chemical functionalities like amino, hydroxyl, carboxyl, and epoxy groups are known to be effective in covalent coupling of proteins and signal molecules. Alternatively, biomolecules may be adsorbed at the surface due to Van-der-Waals dispersion forces, hydrogen bonding, or acid–base interactions (Meyer-Plath, Schroder et al. 2003).

To be used in biomedical applications, improved cellulose integration with the host tissue, to mimic the tissue to be replaced, is required. Chemical surface modifications and incorporation of bioactive molecules are examples of what can be done to make BC an ideal material for reparative tissue engineering. In this context, BC has been modified to further enhance biocompatibility.

1.4.1 Incorporation of bioactive molecules - Recombinant proteins

In order to develop biomaterials that promote specific cellular fates, it is essential to assert control over both the structural properties and biochemical characteristics of these materials, where the use of protein-based biomaterials provides a uniquely powerful approach to the control of macromolecular structure and function (Maskarinec and Tirrell 2005). The expression of recombinant protein polymers promises to expand the use of protein-based materials, both in the investigation of basic cellular processes and in therapeutic applications (Maskarinec and Tirrell 2005). The development of genetic engineering has allowed the design and bioproduction of various protein polymers, which are mainly made from repeating sequences found in natural polymers, such as

elastin, collagen, silks, etc., and selected modifications (Girotti, Reguera et al. 2004).

The combination of polymers with recombinant fusion proteins in tissue engineering constructs can provide a predictable and chemically defined source of ECMs (Nagaoka, Jiang et al. 2010). Although, a prerequisite for the growth of applications based on recombinant fusion proteins is the improvement of the production of larger amounts of functional recombinant proteins.

1.4.1.1 Bioactive molecules for BC modification

Tissue engineering approaches typically employ three-dimensional ECM to engineer new natural tissues from cells. The design of bioactive molecules for tissue engineering intend to mimic the functions of the natural ECM molecules found in tissues, which act as a scaffold to bring cells together to form a tissue and control its structure, and to regulate cell phenotype (Nagaoka, Jiang et al. 2010). To obtain specific cell recognition and adhesion, the bioactive sites from these ECM proteins are often used for the modification of biomaterials.

Indeed, the incorporation of soluble bioactive molecules, such as growth factors and cell-binding peptides into biomaterial carriers, is an important strategy used to achieve biomolecular recognition of materials by cells and allow specific cellular responses (Imen, Nakamura et al. 2009). ECM composition include collagens, laminins, fibronectin, vitronectin, elastin, and integrin binding proteins (Agrawal and Ray 2001). Cell adhesion to ECM is mediated by cell-surface receptors, one important class being the integrins, which bind to short amino acid sequences (RGD sequences) on integrin binding proteins. The amino acid sequence Arg-Gly-Asp (RGD) is recognized for its cellular adhesion function. For this reason, the RGD sequences have gained much attention, and several studies have attempted to isolate specific sequences that promote increased cell adhesion (Agrawal and Ray 2001).

Laminin is a family of large (*Mw* 900,000Da) trimeric basement membrane glycoproteins, which has a structural role in organizing the basement membrane matrix. Several active sites responsible for multiple biological activities such as neurite outgrowth, tumor metastasis, cell attachment and spreading, and angiogenesis were identified in laminin (Kleinman, Klebe et al. 1981; Kanemoto, Reich et al. 1990). The IKVAV (Ile-Lys-Val-Ala-Va) sequence from the α1 chain, is one of the most studied among those active sites, because it can promote neurite outgrowth, tumor metastasis and growth, protease activity, cell adhesion, and angiogenesis (Tashiro, Sephel et al. 1989; Kanemoto, Reich et al. 1990; Malinda, Nomizu et al. 1999). Other sequences, such as YIGSR (Tyr-Ile-Gly-Ser-Arg) on the β1 chain, have different biological activities, including inhibiting angiogenesis, tumor growth and metastasis. Furthermore, sequential screening of peptides has identified several sequences that promote adhesion to a variety of tumor cells. Also, experimental data suggest that a number of additional active sites exist on laminin that could be cell type-specific (Tashiro, Sephel et al. 1989; Malinda, Nomizu et al. 1999). Another bioactive molecule which presents cell type specificity is a peptide that mimics a bioactive domain of neural cell adhesion molecule (NCAM, a cell–cell adhesion molecule of the immunoglobulin superfamily of proteins). The KHIFSDDSSE, the active site of the NCAM, can modulate the astrocyte adhesion, and can be used in improved prostheses for the CNS (Kam, Shain et al. 2002).

Examples of the use of bioactive peptides in the development of biomaterials are described ahead. The RGD sequence within a biocompatible hydrogel of poly[*N*-(2-hydroxypropyl)methacrylamide] (PHPMA) was synthesized by Woerly and colleagues (2001) and used to promote tissue regeneration of injured adult and developing rat spinal cord. The hydrogel provided a structural, three-dimensional continuity across the defect, facilitating the migration and reorganization of local wound-repair cells, as well as tissue development within the lesion. Angiogenesis and axonal growth also occurred within the microstructure of the tissue network, and supraspinal axons migrated into the reconstructed cord segment (Woerly, Pinet et al. 2001). Rafiuddin and Jakakumar (2003) studied the regeneration of injured sciatic nerve with collagen tubes

incorporated with RGD cell-adhesive peptide. Collagen implants were grafted to bridge a gap nerve defect in a rat model. The number of myelinated axons in the regenerated mid-graft of the RGD peptide incorporated groups was higher than on the control. After 90 days of implantation, the mean counts were still higher in the case of RGD peptide group, as compared to controls collagen and autograft groups (Rafiuddin and Jakakumar 2003).

The potential benefits of active peptides in synthesizing materials for the treatment of both peripheral and CNS disorders are tremendous (Zou, Zheng et al. 2009). A biocompatible hydrogel of hyaluronic acid with IKVAV peptide was synthesized by Wei and co-workers (2007). After implantation, the hydrogel formed a permissive interface with the host tissue, with potential to repair tissue defects in the central nervous system by promoting the formation of a tissue matrix and axonal growth, replacing the lost tissue (Wei, Tian et al. 2007). Neural stem cells were seeded in three-dimensional hydrogels coated with IKVAV. The cells began to proliferate after 24 h of incubation, and formed bigger neurospheres at 48 h in experimental group than in control group. The self-assembled hydrogel had good cytocompatibility and promoted the proliferation of neural stem cells (Song, Zheng et al. 2009). Zou and colleagues (2009) synthesized peptide-amphiphile (PA) molecules containing the IKVAV sequence. The results indicated that the self-assembling scaffold containing IKVAV sequence had excellent biocompatibility with adult sensory neurons, promoting neurons adhesion and neurite sprouting and could be useful in nerve tissue engineering (Zou, Zheng et al. 2009).

1.4.1.2 Carbohydrate Binding Modules – CBM3

The use of recombinant proteins containing carbohydrates binding domains (CBMs) fused to the bioactive peptides represents a simple way to make specific adsorption of this peptides on polymer surfaces (Wang, Wu et al. 2006). A CBM is defined as a contiguous amino acid sequence within a carbohydrate-active enzyme with a discrete fold having carbohydrate binding activity (Shoseyov, Shani

et al. 2006). The binding domains have been classified into 43 different families based on amino acid sequence, binding specificity, and structure. The CBMs contain from 30 to about 200 amino acids and exist as a single, double, or triple domain in one protein. Their location within the parental protein can be both C- or N-terminal and is occasionally centrally positioned within the polypeptide chain (Shoseyov, Shani et al. 2006).

Family-III CBM comprises approximately 150 amino acid residues (Tormo, Lamed et al. 1996). They have been identified in many different bacterial enzymes, and in some non-hydrolytic proteins (Poole, Morag et al. 1992; Shoseyov, Takagi et al. 1992; Gerngross, Romaniec et al. 1993; Pages, Belaich et al. 1996) which are responsible for the structural organization of the cellulosomes present in *Clostridium cellulovorans* (CbpA), *Clostridium thermocellum* (CipA and CipB from strains ATCC 27405 and YS, respectively), and *Clostridium cellulolyticum* (CipC) (Tormo, Lamed et al. 1996). Cellobiohydrolase CbhA is a component of the cellulolytic/hemicellulolytic complex termed the 'cellulosome' of the anaerobic thermophilic bacterium *Clostridium thermocellum* and is a typical representative of thermostable multi-modular Ca^{2+}-containing enzymes (Kataeva, Uversky et al. 2003). It is composed of various domains, inclusive a family 3 CBM (CBM3). The most probable role of Ca^{2+} in CBMs is to stabilize the native protein structure (Kataeva, Uversky et al. 2003).

Many CBMs are strictly substrate specific, making them useful as molecular building blocks as well as tools (Hilden and Johansson 2004). The small size also makes CBMs attractive for genetic constructs (Hilden and Johansson 2004). The use of CBMs to deliver peptide signaling molecules such as growth factors, to be used as targeted therapeutics with improved half-life, is relevant for clinical applications (Nishi, Matsushita et al. 1998). Recombinant proteins consisting of growth factor moieties and collagen or fibronectin binding domains were described by various authors, envisaging vascular regeneration. Nishi and colleagues (1998) produced fusion proteins with endothelial growth factor (EGF) and fibroblast growth factor (bFGF) (Nishi, Matsushita et al. 1998).

Ishikawa and co-workers (2006) produced a recombinant protein consisting of the fibronectin collagen-binding domain and the vascular endothelial growth factor 121, which promoted the growth of endothelial cells and induced the expression of Vascular endothelial growth factor receptor 2 (VEGFR-2) on hematopoietic stem cells from bone marrow (Ishikawa, Eguchi et al. 2006). Kitajima et al. described a fusion protein consisting of hepatocyte growth factor (HGF, an angiogenic factor) and a CBM, which promoted the endothelial cells growth and improved the blood vessel formation on collagen sponges *in vivo* (Kitajima, Terai et al. 2007). Pang and colleagues produced a recombinant protein composed of a CBM and a fibroblast growth factor-1 with a type I collagen scaffold as a targeted delivery vehicle for smooth muscle cells and vascular tissue engineering. The fusion protein increased the proliferation of SMCs in the collagen matrix to significantly greater levels and duration than the alone counterparts, suggesting that this protein is an effective strategy for growth factor delivery for vascular tissue engineering (Pang, Wang et al. 2010).

In an attempt to delivery growth factors in nerve repair, Sun and colleagues (2009) demonstrated that the native human NGF-β fused with a collagen binding domain specifically bind to endogenous collagen of the rat sciatic nerves and maintain NGF activity both *in vitro* and *in vivo*. The authors found that, in the rat sciatic nerve crush injury model, the collagen-binding NGF could be retained and concentrated at the nerve injured site, promoting nerve repair and enhanced function recovery following nerve damage (Sun, Kingham et al. 2010). Han and colleagues (2009) also used the collagen binding domain to delivery BDNF for nerve repair and showed that the fusion protein had similar activity in neurite outgrowth in dorsal root ganglia and in PC12 cell survival. The authors used the rat hemisection of spinal cord model, and found that this protein significantly improved the spinal cord injury recovery (Han, Sun et al. 2009).

The CBM3 from *C. thermocellum*, has been shown to bind cellulose, particularly crystalline cellulose (Lehtio, Sugiyama et al. 2003). Genetic constructs involving CBM3 are described in literature in the production of recombinant proteins with specificity to cellulose substrates. The CBM was used by Wierzba et

al. (1995) who described a recombinant protein CBM-RGD which promotes cell adhesion to cellulose (Wierzba, Reichl et al. 1995; Wierzba, Reichl et al. 1995). Andrade *et al*. also produced recombinant proteins containing adhesion peptides (RGD or GRGDY) fused to a CBM with affinity by cellulose (CBM3) and the results showed that the RGD sequence improved the fibroblasts adhesion on BC surfaces (Andrade, Moreira et al. 2008). Fig 1.8 show a scheme of a recombinant protein containing a CBM3 adsorbed to a cellulose membrane.

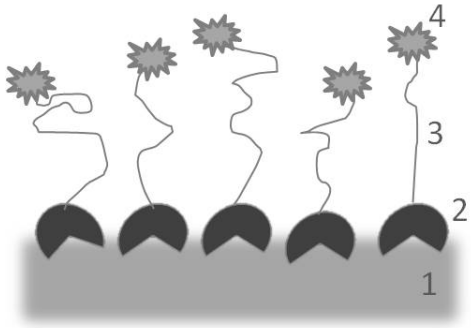

Figure 1.8 Scheme of a cellulose membrane modified trough a carbohydrate binding module conjugated to a bioactive molecule 1) Cellulose; 2) CBM; 3) Linker; 4) Bioactive molecule.

1.4.2 Plasma Technique

In tissue engineering, the bio-integration is the ideal outcome of an artificial implant. This implies that the interaction between the interface of the implant and host tissues do not induce any deleterious effects such as chronic inflammatory response or formation of unusual tissues. Hence, the surface properties are very important regarding the success of the implant. Surface modification of biomaterials is becoming an increasingly relevant method to improve the multifunctionality of biomedical devices, as well its biocompatibility, while obviating the cost and long time required to develop brand new materials. Plasma-surface modification is an effective and economical surface treatment technique for many materials and of growing interests in biomedical engineering (Chu, Chen et al. 2002). Plasma modified materials are well-suited for the control of specific biologic

reactions, and can be engineered as to have appropriate functional groups useful for the immobilization of bioactive molecules (Ratner 1996).

Plasma, often designated the fourth state of matter, consist of a mixture of electrons, ions and neutral particles, although overall it is electrically neutral. The degree of ionisation of plasma is the proportion of atoms that have lost (or gained) electrons. Plasma technology involves the creation of a sustained electrical arc by the passage of electric current through a gas in a process referred to as electrical breakdown (Gomez, Rani et al. 2009). Plasma treatment can easily introduce polarized groups such as hydroxyl, carboxyl, amino and sulfate groups on polymer surfaces using different reaction gases such as air, NH_3, SO_2, CO_2 or other organic compounds (Ma, Mao et al. 2007).

One relevant advantage in the use of plasma treatment is the preservation of the bulk material's chemical and mechanical properties during the process, being this a major concern since many artificial implant and cell culture materials are heat-sensitive polymers (Schroder, Meyer-Plath et al. 2001). If they are processed in the afterglow regime, thermal load on the substrate can be held at a negligible level and bulk material changes can be avoided. Further, plasma activation, as being a gas phase process, reduces the risk of leaving leachable substances on the surfaces (Schroder, Meyer-Plath et al. 2001).

Some examples of successful modification of material by plasma treatment and improved cell-biomaterial interactions can be found in literature. The adhesion of human endothelial cells to polytetrafluoroethylene surfaces, used in vascular prostheses, was improved when the material was treated with nitrogen and oxygen plasma (Dekker, Reitsma et al. 1991). Various polymer surfaces - polyethylene, polypropylene, polystyrene, polyethylene terephthalate and poly(methyl methacrylate) were modified by water vapour plasma discharge treatment, and the high hydroxyl group density produced on the polymers surface had a positive effect on Chinese hamster ovary cell adherence (Lee, Park et al. 1991). The effect of oxygen plasma on the surface modification of different starch-based biomaterials (SBB) and on modulating bone–cells behaviour was described by Alves and colleagues (2006). The authors observed that the adhesion and

proliferation of osteoblast-like cells were enhanced by the plasma treatment on ethylene vinyl alcohol and polycaprolactone materials (Alves, Yang et al. 2007).

Zhao et al. (2006) used NH_3 plasma treatment to incorporate collagen on PDLLA (poly-D,L-lactide) films, and showed that this treatment improved films hidrophillicity and more efficiently enhanced fibroblast cell attachment and proliferation than those films modified by collagen anchorage or only NH_3 plasma treatment (Zhao, Wang et al. 2006). Following this approach, Huang and collaborators used oxygen plasma to incorporate laminin onto the surface of PGLA poly(lactide-co-glycolide) and chitosan films. The authors showed that laminin-modified chitosan membranes significantly increased Schwann cells attachment and affinity for directing peripheral nerve regeneration (Huang, Huang et al. 2007). With the aim to study a polymer treatment for surface engineering for vascular repair, Tajima and colleagues (2007) examined the behavior of endothelial cells seeded on polyethylene surfaces modified by Ar plasma. The authors verified an increased cell adhesion and spreading (Tajima, Chu et al. 2008). Khorasani and colleagues used oxygen plasma treatment to modify the surface of PLA and PLGA films. The results showed that the hydrophilicity increased greatly after O_2 plasma treatment. Cell culture results showed that B65 nervous cell attachment and growth on the plasma treated PLA was much higher than an unmodified sample and PLGA. The surface hydrophilicity and chemical functional groups with high polar component seems to be responsible in the enhanced cell attachment and growth (Khorasani, Mirzadeh et al. 2008).

These are just a few examples of the enhancement of cell-material interaction produced by the plasma treatment of materials, selected among a large number of published works (Dekker, Reitsma et al. 1991; Hsu and Chen 2000; Gupta, Plummer et al. 2002; Hamerli, Weigel et al. 2003; Wan, Yang et al. 2003; Nakagawa, Teraoka et al. 2006; Beaulleu, Geissler et al. 2009; Hauser, Zietlow et al. 2009). However, this is a wide field of research, and each year, more and more materials are processed by plasma and characterized, giving rise to great advances in the development of new biomedical devices and tissue engineering.

1.5 References

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2. Bacterial cellulose modified through recombinant proteins for neuronal cell culture

2.1 Abstract

A wide variety of biomaterials and bioactive molecules has been applied as scaffolds in neuronal tissue engineering. However, creating devices that enhance the regeneration of neural tissues is still a challenge, due the difficulty in providing an appropriate environment for cell growth and differentiation, and active stimulation of nerve regeneration. In recent years, bacterial cellulose (BC) emerged as a promising biomaterial for biomedical applications due its properties, such as high crystallinity, an ultrafine fiber network, high tensile strength and biocompatibility. The small signaling peptides found in the proteins of extracellular matrix are described in the literature as promoters of adhesion and proliferation of several cell lineages on different surfaces. In this work, the peptides IKVAV and KHIFSDDSSE were fused to a carbohydrate-binding module (CBM3) and were used to modify BC surfaces aiming the promotion of neuronal cell adhesion. The recombinant proteins IKVAV-CBM3, *ex*IKVAV-CBM3 and KHIFSDDSSE-CBM3, were successfully expressed in *E. coli,* purified through affinity chromatography, and stably adsorbed to the cellulose membranes. The effect of these recombinant proteins on the adhesion of neuronal and mesenchymal cells was evaluated by MTS colorimetric assay. The results showed that the *ex*IKVAV-CBM3 was able to improve the adhesion of both neuronal and mesenchymal cells, while IKVAV-CBM3 and

KHIFSDDSSE-CBM3 presented only a slight effect on mesenchymal cells, and no effect on the other cells. The MSCs neurotrophin expression by cells growth on BC membranes modified with the recombinant proteins was also verified, in order to search for a microenvironment that promotes neuronal regeneration.

2.2 Introduction

Nerve tissue engineering is a rapidly expanding area of research providing a new and promising approach to nerve repair and regeneration (Prabhakaran, Venugopal et al. 2008). Therefore, the challenge in nerve regeneration is to construct biological substitutes that are capable to maintain a continuous path for regeneration and promoting the infiltration of cells to secrete inductive factors for axonal elongation (Prabhakaran, Venugopal et al. 2008). Besides, the comprehension of neuronal mechanisms and cells behavior in contact with different biomaterials is essential for implementation of advanced prosthesis and complex neural networks (Cecchini, Bumma et al. 2007).

A wide variety of biomaterials and bioactive molecules have been applied in tissue engineering (Huber, Heiduschka et al. 1998; Tong and Shoichet 2001), (Ranieri, Bellamkonda et al. 1994; Bellamkonda, Ranieri et al. 1995; Woerly, Plant et al. 1996; Turner, Kam et al. 1997; Patel, Padera et al. 1998). Among them, biological scaffolds, composed of natural polymers combined with extracellular matrix molecules, have been shown to facilitate the constructive remodeling of several tissues by the establishment of an appropriated environment essential for the regulation of cell processes (Adams and Watt 1993; Badylak, Freytes et al. 2009). In recent years, bacterial cellulose (BC) emerged as a promising biomaterial in tissue engineering due its properties. BC is a glucose linear polymer secreted by *Gluconacetobacter xylinus* composed of a nanofibers network, with appealing properties including high crystalinity, wettability, high tensile strength, moldability *in situ* and simple production (Svensson, Harrah et al. 2004). Despite having identical chemical properties of plant cellulose, BC is produced in a pure form, free of other polymers and its

macromolecular properties and structure are also different (Jonas and Farah 1998; Vandamme, De Baets et al. 1998). These characteristics, beyond its biocompatibility, make the BC an ideal material for tissue engineering constructs.

Previous studies reported the modification of biomaterials surface by immobilization of proteins as a strategy to control and guide, with high selectivity, the interactions between cells and materials (Massia, Stark et al. 2000; Kam, Shain et al. 2002; Hersel, Dahmen et al. 2003). One approach to achieve this goal involves the incorporation of small cell-binding peptides into biomaterials via chemical or physical modification (Shin, Jo et al. 2003). As an alternative to peptide chemical grafts, the use of recombinant proteins containing carbohydrates binding domains (CBMs) fused to the bioactive peptides represents an attractive way to specifically adsorb these peptides on cellulose surface (Wang, Wu et al. 2006; Andrade, Moreira et al. 2008). The CBM3 from the cellulosomal-scaffolding protein A of the *Clostridium thermocellum*, has high affinity to cellulose, particularly to crystalline cellulose (Lehtio, Sugiyama et al. 2003).

A great number of cell adhesion motifs have been identified and used in biopolymer structures to mediate cell attachment. RGD (Arg-Gly-Asp), IKVAV (Ile-Lys-Val-Ala-Val) and KHIFSDDSSE (Lys-His-Ile-Phe-Ser-Asp-Asp-Ser-Ser-Glu) are bioactive cell adhesion motifs found in ECM proteins such as fibronectin, laminin and neural cell adhesion molecule (N-CAM) and are described as promoters of cell adhesion and proliferation in several materials (Massia and Hubbell 1991; Dai, Belt et al. 1994; Woerly, Laroche et al. 1994; Cook, Hrkach et al. 1997; Yamaoka, Hotta et al. 1999; Kam, Shain et al. 2002; Lin, Takahashi et al. 2006; Andrade, Moreira et al. 2008). The IKVAV sequence, located on the C-terminal of the long arm of the laminin α1 chain, was identified as an active site of this protein. This peptide was found to be active in promoting cell adhesion, neurite outgrowth, angiogenesis, collagenase IV production, and tumor growth (Tashiro, Sephel et al. 1989; Nomizu, Weeks et al. 1995). IKVAV peptide has been used in the construction of experimental

nerve guides, since it can mediate cell attachment and neurite outgrowth (Lin, Takahashi et al. 2006). Kam et al. in 2002 presented the KHIFSDDSSE as a novel adhesive peptide that mimics the bioactive domain of N-CAM molecule and specifically binds to astrocyte cells (Kam, Shain et al. 2002). The neural cell adhesion molecule is expressed by astrocytes and mesenchymal cells and has an important regulatory role in the developing nervous system, being present in adult nervous tissue (Cunningham, Hemperly et al. 1987; Sporns, Edelman et al. 1995; Crigler, Robey et al. 2006). The RGD is described as the most effective bioactive peptide and it is often employed to stimulate cell adhesion on various surfaces, due its ability to address more than one cell adhesion receptor and its biological impact on cell anchoring, behavior and survival (Hersel, Dahmen et al. 2003)

Besides increasing the cell-material interaction, an ideal scaffold should maintain the cells viable and functional; in addition cells should be able of secreting growth factors that enhance tissue regeneration. Neural tissue engineering strategies focus on developing scaffolds that artificially generate favorable cellular microenvironments, to promote regeneration, particularly in conjunction with stem cells, has generated promising results (Nisbet, Crompton et al. 2008). The use of stem cells in tissue engineering constructs is a promising strategy, because these cells can express a variety of growth factors important for tissue regeneration and cell differentiation. The transplantation of stem cells can provide support to affected cells by secreting cytokines and neurotrophic factors, which means the creation of a neuroprotective environment (Barzilay, Levy et al. 2006). Neurotrophins such as the nerve growth factor (NGF) are a family of proteins that induce the survival, development and function of neurons (Coumans, Lin et al. 2001) and are common growth factors used to promote neural tissue engineering (Willerth and Sakiyama-Elbert 2007).

The main purpose of this work was to produce recombinant proteins containing a bioactive peptide fused to the CBM3 to functionalize BC surface in order to optimize material biocompatibility. Neuronal and mesenchymal stem

cell (MSCs) adhesion and viability were evaluated on these modified surfaces. The MSCs neurotrophin expression by cells growth on BC membranes modified with the recombinant proteins was also verified, in order to search for a microenvironment that promotes neuronal regeneration.

2.3 Materials and Methods

2.3.1 Production of bacterial cellulose

The pellicles of bacterial cellulose were produced by the *Gluconacetobacter xylinus* (ATCC 53582) cultured in Hestrin & Schramm medium, into 24-wells polystyrene plates (800 µl per well), for 4 days at 30 ^{o}C, in static culture. The membranes were purified with 2% Sodium dodecyl sulfate (SDS) overnight, then washed with distilled water until the complete removal of SDS and immersed in a 4% NaOH solution, shaking for 90 min at 60 ^{o}C. After neutralized, the pellicles were autoclaved in Phosphate buffered saline (PBS) and stored at 4 ^{o}C.

2.3.2 Cloning, expression and purification of recombinant proteins

The In this work, we produced 3 recombinant proteins: IKVAV-Linker-CBM3, KHIFSDDSSE-Linker-CBM3 and *ex*IKVAV-linker-CBM3 (CSRARKQAASIKVAVSADR-CBM3) corresponding to the extended amino acid sequence based on the proteolytic laminin fragment PA-22 containing the sequence IKVAV (Sephel, Tashiro et al. 1989; Tashiro, Sephel et al. 1989; Mackay, Gomez et al. 1994; Nomizu, Weeks et al. 1995; Adams, Kao et al. 2005). The linker sequence contains 40 aminoacids. The cloning, expression and purification of recombinant proteins were developed following protocol described by Andrade and colleagues (Andrade, Moreira et al. 2008). Briefly, coding sequences were obtained by PCR using the pET21a-CBM3 vector and the primers shown in Table 2.1, including *Nhe*I and *Xho*I restriction sites (in bold). The PCRs condition used were: preheating at 95 ^{o}C for 2 min, 40 cycles

at 95 ^{o}C for 45 s, 56 ^{o}C for 45 s and 72 ^{o}C for 45s, followed by an elongation cycle at 72 ^{o}C for 10 min. The PCR products were analyzed by agarose gel, purified (Quiagen), digested with *Nhe*I and *Xho*I restriction enzymes, and cloned into the expression vector pET21a (Novagen), previously digested with the same restriction enzymes. This vector includes a C-terminal His6-tag in the recombinant proteins to allow the purification by immobilized metal ion affinity chromatography (IMAC) using a 5 ml nickel His-Trap column (GE Healthcare). The *E. coli* XL1 Blue (Stratagene) was used as cloning strain and the integrity of cloned PCR products was verified by DNA sequencing (Sanger, Nicklen et al. 1977).

Table 2.1 Primers used for cloning the DNA sequences encoding the peptides in fusion with CBM3

Construct	Primers
*ex*IKVAV-LK-CBM3	*Forward* 5' CTA **GCT AGC** TGT TCA AGG GCT AGG AAG CAG GCT GCT TCA ATA AAG GTA GCT GTA TCA GCT GAT AGG ACA CCG ACC AAG GGA G 3'
IKVAV-LK-CBM3	*Forward* 5' CTA **GCT AGC** ATA AAG GTA GCT GTA ACA CCG ACC AAG GGA G 3'
KHIFSDDSSE-LK-CBM3	*Forward* 5' CTA **GCT AGC** AAA CAT ATA TTT TCA GAT GAT TCA TCA GAA ACA CCG ACC AAG GGA G 3'
	Reverse 5' CAC **CTC GAG** TTC TTT ACC CCA TAC AAG AAC 3'

2.3.3 Production and purification of recombinant proteins

Recombinant proteins were produced using the *E. coli* BL21 (DE3) cells transformed with the expression vectors containing the different coding sequences, pET21a-CSRARKQAASIKVAVSADR-LK-CBM3, pET21a-IKVAV-LK-CBM3 and pET21a-KHIFSDDSSE-LK-CBM3, were grown at 37°C, in LB medium supplemented with ampicillin (100 µg/ml). Cultures were induced with IsoPropyl β-D-1-ThioGalactopyranoside (IPTG, Invitrogen) at 1 mM. Five hours

after induction, the cells were separated from the culture medium by centrifugation (13 000 g, 10 min) and resuspended in buffer A (20 mM Tris, 20 mM NaCl, 5 mM $CaCl_2$, pH 7.4) and then lysed by sonication. The soluble and insoluble fractions were separated by centrifugation (15 000 g, 4 °C, 30 min). The purification was made by affinity chromatography, using a HisTrapTM HP (GE health care). For that, imidazole was added to the cell lysated (40 mM final concentration) and the pH was adjusted to 7.4 before its application on the nickel column. After purification, proteins were dialyzed against the buffer A, sterilized by filtration (0.22 µm) and stored at -20 °C, prior to use. Recombinant proteins were analyzed by 12% SDS-PAGE (SDS – polyacrylamide gel electrophoresis) stained with Coomassie blue.

2.3.4 Adsorption assay

The wells of a 24-well polystyrene plate were covered with BC pellicles, the recombinant proteins were added to the wells (0.25 mg protein per well) and left adsorbing at 4 ^{o}C, overnight. The non-adsorbed proteins were collected and the membranes were washed three times with Buffer A to remove the non-adorbed protein. Then, the membranes were washed three times with Buffer A containing 1% SDS to remove the adsorbed protein, and collected. The initial protein solution, the non-adsorbed proteins (supernatant fraction) and the adsorbed protein fraction were analyzed by SDS-PAGE.

2.3.5 Cell culture

SH-SY5Y human neuroblasts, N1E-115 rat neuroblasts, Human Microvascular endothelial cells (HMEC-1), rat Pheochromocytoma (PC12), rat Mesenchymal stem cells (MSCs), and mice astrocytes were maintained under standard tissue culture condition (37 ^{o}C, 5% CO_2, 95% humidified air). SH-SY5Y cells were cultured in a complete medium containing 1:1 Dulbecco's Modified Eagle Medium (DMEM; Gibco) and Ham Nutrient Mixture (Ham F-12;

Sigma) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% Penicillin/Streptomicin; N1E-115 were cultured in DMEM supplemented with 10% FBS and 1% Penicillin/Streptomicin. HMEC-1 and PC12 were cultured in RPMI with 10% and 15% of FBS (inactivated), respectively, and 1% Penicillin/Streptomicin. Rat MSCs (rMSCs) were isolated from femur and tibias of adult Winstar rats as previously described (Jiang, Lv et al. 2010) and cultured in DMEM supplemented with 20% FBS and 1% Penicillin/Streptomicin. Astrocytes were isolated according to (Blondeau, Beslin et al. 1993) and cultured in DMEM with 10% of FBS and 1% Penicillin/Streptomicin.

2.3.6 Cell adhesion and viability on recombinant proteins coated surfaces

Cell adhesion was determined by mitochondrial activity through a MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] colorimetric assay, performed as follows: the proteins were added to a 24-well polystyrene plate (0.25 mg protein per well) covered with the BC pellicles. Plates were incubated overnight at 4 ^{o}C. The unbound proteins were removed and the BC pellicles washed with PBS. Cells were then seeded in serum-free medium (excepted SH-SY5Y cells) at a density of 6 $\times 10^4$ cells/well on BC pellicles. After 2 h, the wells were washed with PBS and transferred to new wells where complete medium was added, and the MTS was performed. The control used was the BC membranes treated only with buffer A. The cell adhesion experiments were run in triplicate at two separated times.

2.3.7 Live and Dead assay

The viability of the cells cultured on BC membranes coated with the recombinant proteins for 10 days was determined through the live/dead assay. The LIVE/DEAD® Viability/Cytotoxicity Kit for mammalian cells (Invitrogen) provides two-color fluorescence cell viability assay based on the determination

of live and dead cells with two probes that measure intracellular esterase activity and plasma membrane integrity. 100 µL of a solution of calcein and ethidium homodimer-1 in sterile PBS were added to the wells, incubated for 30 to 45 minutes at 37ºC, 5% CO2, 95% humidified air and visualized in a fluorescence microscope on the BC membranes.

2.3.8 Enzyme-linked immunosorbant assay (ELISA)

To determine the levels of expressed neurotrophins by rMSCs, the concentration of NGF was measured, using a commercial ELISA kit (Promega) according to the manufacturer's instructions. The cells were cultured in DMEM 2% FBS on BC membranes treated with recombinant proteins. BC without recombinant proteins and polystyrene plate were used as assays controls. The supernatant of the cells was removed at 3, 6 and 13 days and kept under -80 ºC, and fresh medium was added to the wells. The levels of neurotrophins were calculated using the standard curve. Samples and standards were run in duplicate.

2.3.9 Statistical analysis

All results are presented as mean ± standard deviation. Multiple comparisons were performed by ANOVA followed by Bonferronis secondary test for significance between experimental conditions and control conditions ($p < 0.05$).

2.4 Results

In this study recombinant proteins were expressed using an *E. coli* expression system and purified in order to functionalize BC membranes, improving the adhesion of neuronal cells and biological response of neural implants. The peptides used are described in literature as promoters of

adhesion and proliferation of different cell lineages, and they were fused to a carbohydrate-binding module, the CBM3, to adsorb easily these peptides to the BC.

The *ex*IKVAV-CBM3, IKVAV-CBM3 and KHIFSDSSE-CBM3 proteins were successfully expressed in the soluble fraction of *E. coli* and purified through affinity chromatography. Figure 2.1 shows the analysis of purification process of the recombinant proteins, by 12% SDS-PAGE stained with Coomassie blue.

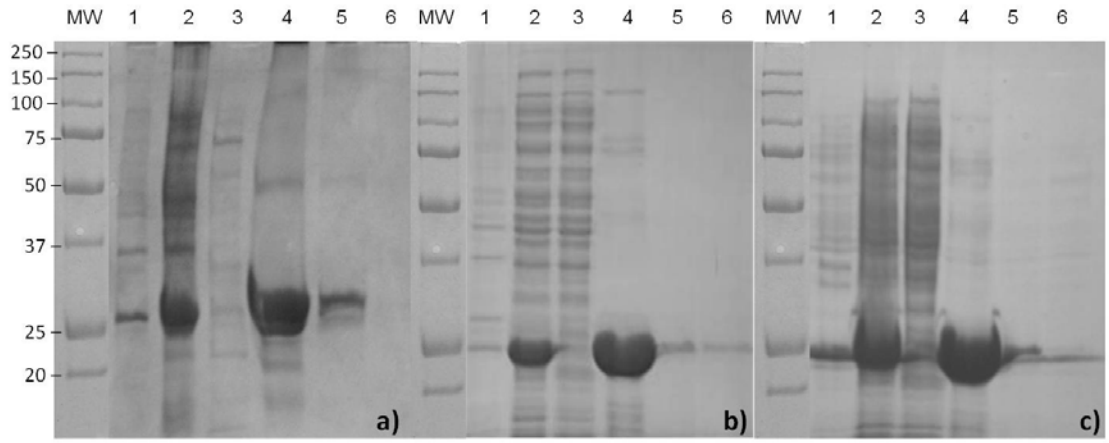

Figure 2.1 SDS-PAGE analysis of expressed and purified recombinant proteins. 1- Molecular weight marker (Biorad); a) *ex*IKVAV-CBM3; b) IKVAV-CBM3; c) KHIFSDDSSE-CBM3.1- Pellet; 2- Supernatant; 3- Flow fraction; 4-Purified protein fraction 1; 5- Purified protein fraction 2; 6- Cleaning solution.

2.4.1 Adsorption assay

The modification of BC surface was achieved through adsorption of the CBM3 to cellulose. This interaction is stable and desorption occurred only in the presence of buffer containing 1% SDS, as shown in figure 2.2.

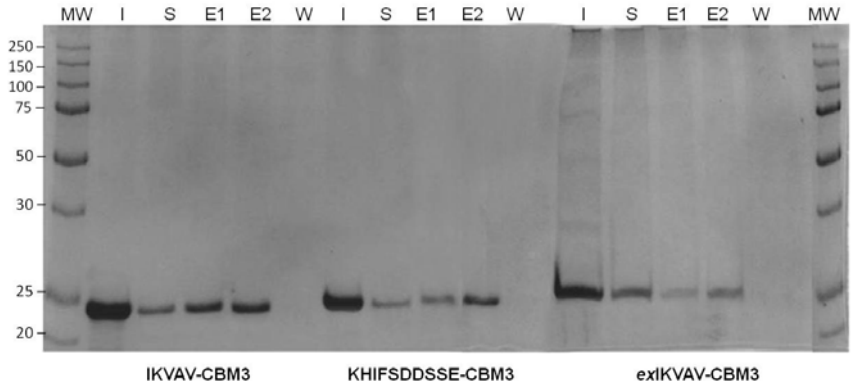

Figure 2.2 SDS-PAGE analysis of recombinant protein adsorption on BC membranes. MW - Molecular weight marker (Biorad); I – Initial recombinant protein (0.5 mg/ml); S – Supernatant containing the non-adsorbed protein; E – Elution fraction of recombinant proteins in buffer containing SDS; W – washing fraction without SDS.

2.4.2 Cell adhesion and viability

Figure 2.3 shows the MTS results. The recombinant protein *ex*IKVAV-CBM3 increased significantly the adhesion of all cells lineages tested, but the effect depended on the cell type. This protein improved almost 100% of cell adhesion of PC12 cells. The RGD-CBM3 protein also improved the adhesion of N1E-115 and mesenchymal cells, revealing a cell specific behavior. On the other hand, the IKVAV-CBM3 and KHIFSDDSSE-CBM3 only presented a slight effect on mesenchymal cell adhesion. The KHIFSSDSSE also presented a slight effect (5%) on astrocyte cell adhesion compared to control.

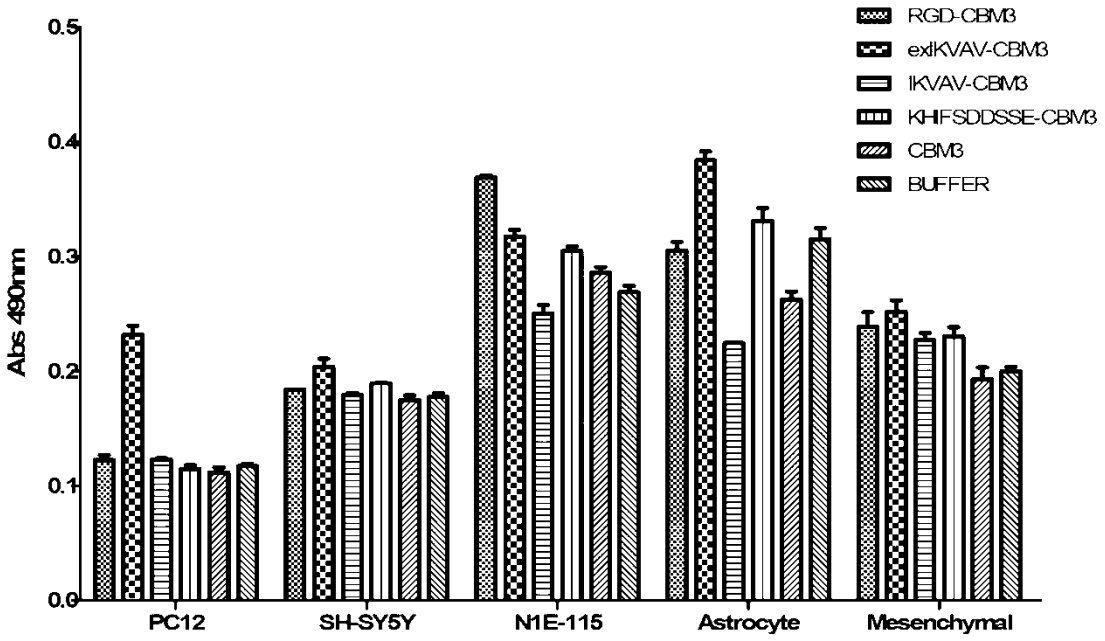

Figure 2.3 MTS assays of PC12, SH-SY5Y, N1E-115, astrocytes and mesenchymal cells seeded on BC pellicles coated with recombinant proteins. Cells were able to adhere in BC surfaces for 2h. The control was BC pellicle treated with Buffer A without recombinant proteins.

The presence of serum in the culture medium represented a relevant factor in cell attachment. The SH-SY5Y cell adhesion only occurred in medium-containing serum, while the adhesion of the other cell types was significantly increased in serum-free medium.

Figure 2.4 shows fluorescence images of PC12 and mesenchymal cells on BC membranes coated with the recombinant protein *ex*IKVAV-CBM3, after 2 weeks in culture. The results show that both cell types remained adhered and alive (stained in green) on the BC, with practically no dead cells (stained in red), but cells showed a rounded morphology. It can be seen that, in control wells, there are fewer cells attached, mainly in PC12 culture. These results are in agreement with adhesion results, where *ex*IKVAV-CBM3 improved strongly the adhesion of PC12 cells, and improved mesenchymal adhesion too, compared to control.

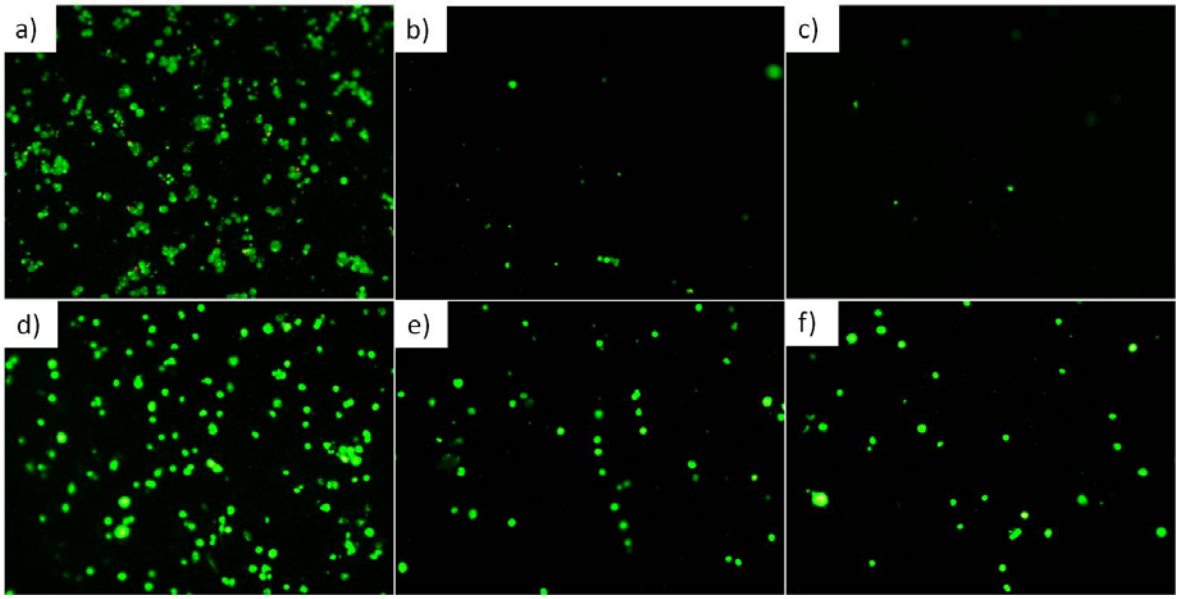

Figure 2.4 Images showing the live/dead assay of PC12 (a,b,c) and mesenchymal (d, e, f) cells cultured for 2 weeks on BC coated with *ex*IKVAV-CBM3 (a and d); CBM3(b and e); Buffer (c and f).

2.4.3 Neurotrophin expression

To investigate the neurotrophin expression of rMSCs on BC coated with the recombinant proteins, we used ELISA kits to quantify the neurotrophins released to the culture medium. The results showed that NGF is produced by the rMSCS and is released to the rMSCs culture medium after 3 and 6 days. Figure 2.5 shows the NGF expression. In agreement with the higher cell adhesion observed on BC coated with the recombinant proteins, the RGD-CBM3 and *ex*IKVAV-CBM3 allowed a higher amount of NGF in the supernatant, as compared to CBM3 and buffer. As expected, cells on polystyrene showed a higher amount of NGF in supernatant, also caused by the number of adhered and proliferating cells on this material (data not shown). Moreover, the expression of NGF was higher at the 13th day rather than at the 3rd and 6th days. At day 6, a slightly decrease of the NGF in supernatant compared to day 3 was observed.

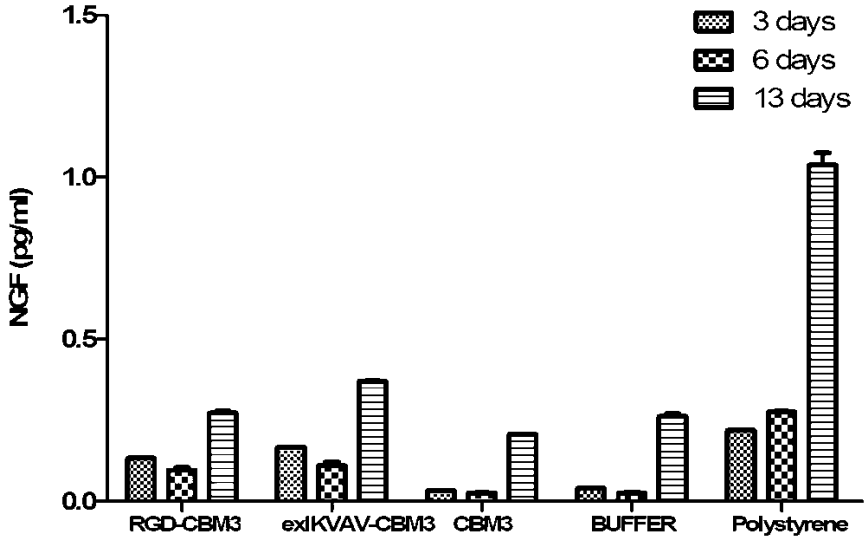

Figure 2.5 ELISA results of NGF expression by rMSCs on BC coated with recombinant proteins and polystyrene.

2.5 Discussion

One of the main challenges of tissue engineering technologies is the production of materials designed to act as adequate scaffold for the growing of cells and tissues (Girotti, Reguera et al. 2004). Improvement of cell adhesion may be achieved by the immobilization of ECM adhesion proteins, or of its signaling motifs, onto the biomaterials surface (Hersel, Dahmen et al. 2003). Attempting to select cell attachment and elicit specific cell responses, we produced different recombinant proteins with the bioactive peptides IKVAV and KHIFSSDSSE. Different cell lineages were used to evaluate the efficacy of these bioactive peptides conjugated with CBM3 on the functionalization of BC membranes for its application as scaffolds in neuronal tissue engineering.

It is know that the use of short peptides containing the signaling motifs instead of the whole adhesive proteins (laminin, fibronectin), have advantages including the ease and reproducibility of synthesizing peptides, as compared with isolating ECM molecules from a natural source (Hubbell 1999). However,

this approach have limitations, such as the biological activity of short peptide sequences is often substantially lower than that of the complete protein, owing at least partially to the absence of complementary domains that are involved in cell receptor binding (Yang, Roach et al. 2001; Boontheekul and Mooney 2003). In fact, in this work, the recombinant protein *ex*IKVAV-CBM3 increased significantly the adhesion of all cells lineages tested, but the effect was dependent on the cell type. The MTS results showed an improvement of almost 100% in cell adhesion for PC12 cells and 30% of mesenchymal stem cells. The RGD-CBM3 protein also improved the adhesion of N1E-115 and mesenchymal, revealing a cell specific behavior. On the other hand, the IKVAV-CBM3 and KHIFSDDSSE-CBM3 only presented a slight effect on mesenchymal cell adhesion. Maybe, the use of the smallest recognition sequence (IKVAV or KHIFSDDSSE) on the protein construction led to a weak interaction between the active peptides and the receptors on the cell surface. In this context, some studies showed that using an extended peptide containing the IKVAV sequence, such as CSRARKQAASIKVAVSADR, it is possible to increase the cell-protein interaction (Klein, Scholl et al. 1999; Tong and Shoichet 2001; Shaw and Shoichet 2003; Massia, Holecko et al. 2004; Lin, Takahashi et al. 2006; Lu, Bansal et al. 2006; Moreira, Andrade et al. 2008). Shaw and Soichet (2003) compared the cell adhesion on modified surfaces with the laminin-derived cell adhesive peptides CIKVAV and CQAASIKVAV. The surfaces modified with extended peptide sequences CQAASIKVAV demonstrated a greater number of cells attached compared to that modified with the shorter peptide sequences, indicating that the extended peptides mimic more closely the three-dimensional conformation that this peptides maintain in laminin (Shaw and Shoichet 2003). Andrade et al. (2008) also described differences on cell adhesion dependent of amino acids flanking the RGD sequence in recombinant proteins, where RGD-CBM and GRGDY-CBM had different efficacy on fibroblast cell adhesion. Moreover, the surface where the proteins are adsorbed can influence in the exposition of the bioactive site, leading to different patterns of cell attachment (Wierzba, Reichl et al. 1995; Andrade, Moreira et al. 2008). For example, the effect of *ex*IKVAV-CBM3 protein was also assessed on polystyrene (data not

shown), however no effect on the cell adhesion was observed, while the RGD-CBM3 had a higher cell adhesion on polystyrene.

The KHIFSSDSSE presented only a slight effect (5%) on astrocyte cell adhesion compared to control (Buffer A), which was not expected, considering that KHIFSDDSSE sequence is related to specific binding to astrocytes (Kam, Shain et al. 2002; Lu, Bansal et al. 2006). However, it is described that the topology and roughness of the surface and the conformation of the cell-adhesion molecules can reduce the effective density of exposed adhesive biomolecules accessible to the cell receptors and can reduce significantly the affinity of the receptor-ligand binding.

It is known that in physiological settings, cells interpret signals from the ECM and different cell types interact with different matrix proteins (Orner, Derda et al. 2004). Therefore, it is not surprising that the intrinsic conditions of the used *in vitro* system, among them cell line, culture medium, presence of serum, roughness and topography of material, structure and conformation of peptide have a strong influence on the pattern of cellular behavior, as observed in this work.

The survival of anchorage dependent cells, such as MSCs requires a support matrix, because in the absence of cell-matrix interactions, these types of cells undergo apoptosis (Frisch and Ruoslahti 1997; Ishaug-Riley, Crane-Kruger et al. 1998; Nuttelman, Tripodi et al. 2005). Thus, when designing hydrogel niches to serve as synthetic extracellular matrix environments, preservation of matrix–cells receptors interactions is critical to promote long-term cell survival and function (Nuttelman, Tripodi et al. 2005). In order to verify the survival of cells on BC modified with the recombinant protein *ex*IKVAV-CBM3 the live and dead assay was performed. The results showed that PC12 and mesenchymal cells remained adhered and viable after 2 weeks on BC coated with *ex*IKVAV-CBM3 protein. However cells maintained a rounded morphology, without signals of cell spreading, proliferation or differentiation, in accordance with results previously described with other materials, such hydrogels and nanofibers gel (Wu, Zheng et al. 2006; Wu, Zheng et al. 2010).

Wu and collaborators (2010) showed that the self-assembly peptide IKVAV promoted the cell adhesion and viability of bone MSCs, but exerted no influence on the proliferation of the MSCs. Also, IKVAV ligand on poly(ethylene glycol) (PEG) hydrogels showed to increased hMSC viability on nondegradable hydrogel, but not in degradable hydrogel, and alone was not capable to influence cell spreading (Jongpaiboonkit, King et al. 2009).

It has recently been demonstrated that MSCs, even without any induction, are able to secret neurotrophins, providing a natural source of these molecules, which can be used in tissue engineering (Jiang, Lv et al. 2010). Our results showed that NGF is produced by the rMSCS seeded on BC membranes and released to the culture medium after 3, 6 and 13 days. RGD-CBM3 and *ex*IKVAV-CBM3 proteins secreted a higher amount of NGF to the supernatant, probably due to the higher initial number of cells adhered to BC treated with those proteins. Cells on polystyrene had a higher amount of NGF in supernatant not only caused by the initial adhered cells, but also because of cell proliferation on this material (data not shown). The neurotrophin release is important in the survival or function of damaged cells within the local tissue, providing a signal that elicits cell proliferation or migration within the tissue region (Saltzman and Olbricht 2002). Therefore, our results indicate that BC modified functionalized with recombinant proteins represent a good scaffold to tissue engineering because, besides increasing cell adhesion, cell viability is mantained and allows the rMSCs to express neurotrophic factors necessary to create a suitable environment to tissue regeneration.

2.6 Conclusion

BC is a promising biomaterial to be used as scaffold in tissue engineering applications but, as in most scaffolds, it is still necessary to increase the interaction of cells with the material to obtain a matrix that maintains the growth, differentiation and selectivity of different cell types. The recombinant peptides were successfully expressed in *E. coli* and adsorbed in a stable way to the

cellulose membranes. The recombinant protein *ex*IKVAV-CBM3 strongly improved PC12 and mesenchymal cell adhesion, indicating that this recombinant protein can be used in BC scaffolds for neural tissue engineering applications.

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3. Surface modification of bacterial cellulose by nitrogen-containing plasma for improved interaction with cells

Adapted from (2010) **Carbohydrate Polymers**, 82(3): 692-698

3.1 Abstract

Bacterial cellulose (BC) membranes were modified with nitrogen plasma in order to enhance cell affinity. The surface properties of the untreated and plasma modified BC (BCP) were analyzed through contact angle measurements, X-ray photoelectron spectroscopy (XPS) and scanning electron microscopy (SEM). The effect of the plasma treatment on the adhesion of microvascular (HMEC-1), neuroblast (N1E-115) and fibroblast (3T3) cell lines was analyzed. The nitrogen plasma treatment did not increase the wettability of the material, but increased the porosity and surface chemistry, as noticed by the presence of nitrogen. XPS analysis revealed the stability of the modified material along time and autoclave sterilization. The cell adhesion and proliferation of HMEC-1 and N1E-115 cells was significantly improved in the BCP, in contrast with the 3T3 cells, revealing a cell-specific effect. This work highlights the potential of plasma treatment for the modification of the BC surface properties, enhancing its potential for biomedical applications.

3.2 Introduction

The surface characteristics play a vital role in the *in vivo* performance of biomaterials. The fate of implants is determined by the interactions - to a large extent cell specific (Chu, Chen et al. 2002) - between the biomaterial and tissues. Polymeric materials do not always possess the specific bioactivity required to promote suitable interaction with cells, thus methods to enhance biocompatibility are required (Wang, Robertson et al. 2004; Ma, Mao et al. 2007). The surface properties of a scaffold, such as wettability, topography, chemistry, surface charge, the presence of hydrophobic and hydrophilic domains, density and conformation of functional groups, all play a crucial role in the cell-material interaction (Vesel, Junkar et al. 2008). The control of cell adhesion on the polymer substrate, and therefore the ability to guide proliferation, migration and differentiation, is highly desirable and a central issue in the development of scaffolds for tissue engineering (Lucchesi, Ferreira et al. 2008).

Surface properties may be altered by plasma-treatment techniques. The modulation of the effects obtained is possible through control of operational parameters, including the gas used, reaction conditions (power, pressure and exposure time) and the reactor geometry (Wang, Lu et al. 2006). Plasma technique is a convenient method to modify the surface properties of polymeric materials, keeping intact their bulk properties. Furthermore, it is an easy way to introduce the desired groups or chains onto the surface of materials with complex shape; being conducted in vacuum, the treatment is pervasive, which is an advantage in the case of scaffolds with interpenetrating porous structures often used for tissue engineering purposes (Yang, Bei et al. 2002).

Bacterial cellulose (BC) is a glucose linear polymer secreted by *Gluconacetobacter xylinus* in the form of nanofibers network, with appealing properties for tissue engineering, including high crystallinity, wettability, high tensile strength, moldability *in situ* and simple production (Svensson, Harrah et al. 2004). Although chemically identical to plant cellulose, BC is obtained free of other polymers and its macromolecular properties and structure are different

(Vandamme, De Baets et al. 1998). Beyond these characteristics, the BC is biocompatible, which makes it ideal for the construction of biomedical devices (Helenius, Backdahl et al. 2006).

In this work, we aimed at modifying BC surfaces with plasma, in order to enhance its cell affinity. Cell adhesion and viability of different cell lineages were evaluated on BC surfaces before and after modification with nitrogen-containing plasma. The effect of plasma treatment, reported in this work for the first time with BC, was accessed through contact angle measurements, scanning electron microscopy (SEM) and X-ray photoelectron spectra (XPS).

3.3 Material and Methods

3.3.1 Bacterial cellulose production

The BC membranes were produced by growing the *Gluconacetobacter xylinus* purchased from the American Type Culture Collection (ATCC 53582) in Hestrin-Schramm medium, pH 5.0. The medium was inoculated and added to polystyrene petri dishes (20 ml per plate), for 4 days at 30 ^{o}C, in static culture. The membranes were purified with 2% Sodium Dodecyl Sulfate (SDS) for 12 h at 60 ^{o}C, washed with distilled water until complete removal of SDS and immersed in a 4% NaOH solution - gently shaken - for 90 min at 60 ^{o}C. After neutralization, the pellicles were autoclaved in distilled water and lyophilized.

3.3.2 Cell culture

Human Microvascular Endothelial Cells (HMEC-1) were cultured in RPMI 1640 medium (Invitrogen Life Technologies, UK), supplemented with 10% FBS (Invitrogen Life Technologies, UK), 1% penicillin/streptomycin (Sigma), 1.176 g/L of sodium bicarbonate, 4.76 g/L of Hepes, 1 mL/L of EGF and 1 mg/L of hydrocortisone > 98% (Sigma). 3T3 mouse embryo fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma) supplemented with 10%

CBS (Invitrogen Life Technologies, UK), 1% penicillin/streptomycin (Sigma). N1E-115 (rat neuroblasts) were cultured in DMEM (Sigma) supplemented with 10% fetal bovine serum (FBS; Gibco), 1% penicillin/streptomycin (Sigma). All cultures were maintained at 37 °C, in atmosphere of 5% CO_2 and 95% humidified air.

3.3.3 BC Surface Modification by Plasma Treatment

The lyophilized bacterial cellulose sheets were treated in a plasma reactor, fed with N_2 (100%). The plasma reactor used consists of a reaction chamber, a vacuum system, a system of power and data acquisition. In addition to two electrodes and an adjusting ring, the plasma chamber also included a glass cylinder 400 mm in length and 320 mm in diameter, generating a total volume of 0.32 m^3. The ends of the tube are sealed by two stainless steel flanges. The connection of bottom flange held vacuum, pressure sensors and thermocouples. The power supply has an output continuously adjustable up to 1500 V DC and current of 2 A. The samples were fixed on the inside camera, using an adjustment ring, and placed between the two electrodes, at a distance of 4 cm from the cathode, as described previously (Costa, Feitor et al. 2006). This distance was necessary to avoid thermal alterations on the surface during processing, once the cathode reached temperatures above 150 ^oC during previous experiments. All treatments were performed under the same conditions: time (30 min); voltage (425 V), current (0.20 A), N_2 Flow (10 sccm), pressure (4 mbar). The cathode temperature was measured and controlled in the control panel.

3.3.4 Determination of contact angles - wettability

Water contact angles were measured using a face contact angle meter (OCA 20, Dataphysics, Germany). The contact angle of the untreated and treated bacterial cellulose surfaces was measured by the sessile drop method

(Kwok and Neumann 1999), in which a 2 *µ*l droplet of ultra pure water (Milli Q) was placed on a horizontal BC surface and observed with a face contact angle meter. The angle formed by the tangent of the droplet with the surface was measured by image analysis.

3.3.5 Scanning electron microscopy

The BC samples were sputter-coated with gold and examined by scanning electron microscopy (SEM). The analyses were performed on a scanning electron microscope (Nova NanoSEM 200, The Netherlands) using an accelerating voltage of 5 kV.

3.3.6 Analysis of X-ray photoelectron spectra (XPS)

The XPS analysis was performed using an ESCALAB 200A, VG Scientific (UK) with PISCES software for data acquisition and analysis. For analysis, an achromatic Al (Kα) X-ray source operating at 15 kV (300 W) was used, and the spectrometer, calibrated with reference to Ag 3d5/2 (368.27 eV), was operated in CAE mode with 20 eV pass energy. Data acquisition was performed at a pressure below 1.E-6 Pa. Survey scan spectra were obtained at a pass energy of 50 eV, while for C 1s, O 1s and N 1s individual high-resolution spectra were taken at a pass energy of 20 eV and a 0,1 eV energy step. Spectra analysis was performed using peak fitting with Gaussian-Lorentzian peak shape and Shirley type background subtraction (or linear, taking in account the data). The binding energy (eV) scales were referenced to the hydrocarbon component (C-C) in the C 1s spectra at 285 eV. Ageing of the plasma-treated samples was also observed by XPS analysis. In this case, the characterization was carried at different time intervals after plasma treatment; the samples were stored at room temperature.

3.3.7 Cell adhesion and proliferation assay

The mitochondrial activity of the cultured cells was determined using a colorimetric assay, which is related to cell viability. The MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay was performed as follows: the bacterial cellulose sheets were cut into circular discs (15mm diameter), sterilized by autoclaving and placed in 24-well tissue culture polystyrene plates; the BC sheets were kept at the bottom of the plate using homemade Teflon hollow cylinders, designed as to fit the wells of the polystyrene plate. Afterwards, 500µl of cell solution in culture medium was added to the wells ($6x10^4$ cells/well). Two hours after the addition of cells, the wells were washed with PBS and complete medium was added. Then, the MTS method was applied to quantify viable cells adsorbed on the BC membrane. The experimental time periods analyzed were 2 h, 24 h and 48 h. The cell adhesion experiments were run in two independent assays, each one performed in triplicate. The plates were incubated for 2 h with MTS reagent, and then 100 µl of each well were transferred to a new plate and read on a Micro Elisa reader (Biotech Synergy HT), with a wavelength of 490 nm.

3.3.8 Statistical Analysis

Experimental data were analysed statistically using one way Analysis of Variance (ANOVA) followed by Tukey test with $p < 0.05$ (*) considered as statistically significant. All statistical analyses were performed with the software program SigmaStat (SigmaStat 3.1, 2004, Excel, 2007, USA).

3.4 Results and Discussion

The modification of surfaces using plasma techniques are becoming increasingly common in biomaterials engineering. The most important advantage of plasma surface modifications is the ability to selectively change the surface properties, improving biocompatibility and mimicking the local tissue

environment without altering the bulk attributes. Plasma thus provides a versatile and effective means to modify surfaces, enhancing the physicochemical properties and optimizing the biofunctionality (Chu, Chen et al. 2002). The nitrogen plasma is often used to modify metals, polymers and polymeric membranes, aiming the introduction of amino groups in the polymer surface and therefore, changing its polarity, reactivity and wettability (Kull, Steen et al. 2005; Charpentier, Maguire et al. 2006).

The bacterial cellulose membranes were submitted to nitrogen plasma treatment with the purpose of enhancing the cell-material interactions. Wettability, evaluated through the measurement of the contact angle of a liquid on a surface, is a sensitive way to detect surface modifications (Charpentier, Maguire et al. 2006). Furthermore, it is a measure of the hydrophilic/hydrofobic character of a material, a relevant property regarding biocompatibility, since it has a major influence on protein adsorption and interaction with cells. In this work, the wettability of the plasma-treated (BCP) and untreated bacterial cellulose (BC) was evaluated by water contact angle measurements. The results showed a slight increase in contact angles in the BCP membranes, the effect of sterilization – also analysed in this study - being not significant. Overall, a slight reduction in the wettability (lower hydrophilicity) follows from the plasma treatment (Fig 3.1). According to Deslandes (1998), this behaviour is not directly related with the plasma treatment. In the work performed by this author, the contact angle of pure cellulose paper sheets increases (as in this work), in control experiments were the material is processed without ignition of the plasma; indeed, these samples were significantly more hydrophobic than the untreated cellulose. The reduction in hydrophilicity of the cellulose samples processed in the plasma chamber - without plasma – was assigned to the removal of physisorbed water and other volatile molecules, which tend to render the surface hydrophilic (Deslandes, Pleizier et al. 1998).

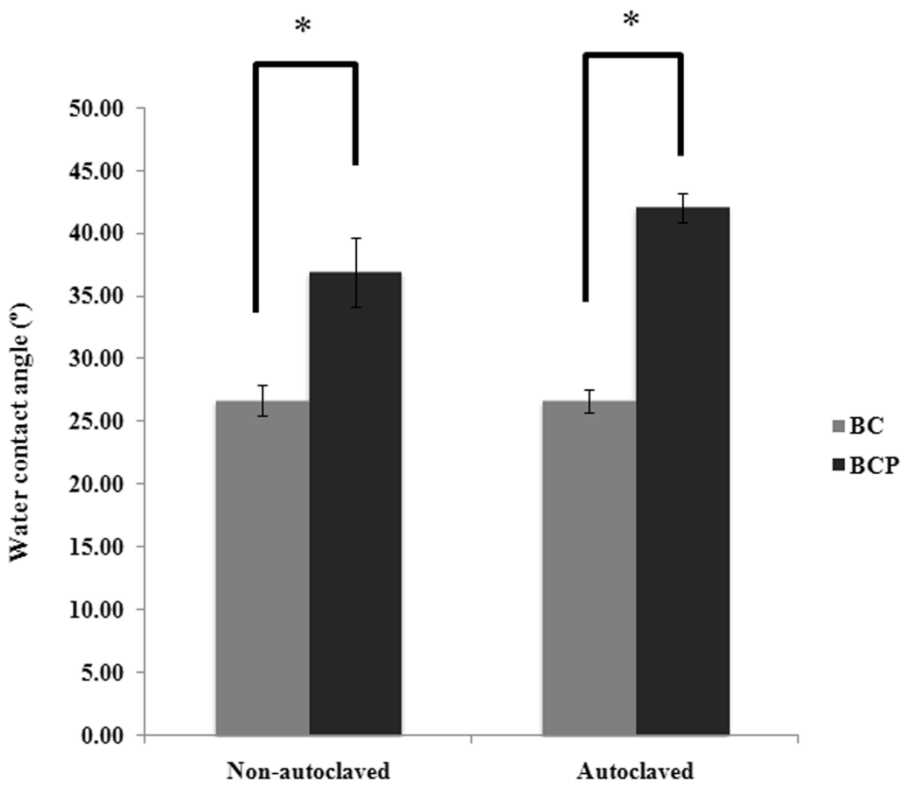

Figure 3.1 Effect of nitrogen plasma treatment on the water contact angle on BC membranes. * $p < 0.05$ considered as statistically significant.

The pressure applied in the plasma treatment may influence the final surface hydrophilicity of the material. Wang (2006) studied the effect of oxygen and nitrogen plasma treatment on Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) films. The authors observed that the contact angles decrease slightly with the exposure time, for both oxygen and nitrogen plasma treatments. However, when the pressure of the chamber increases, the contact angle decrease for the oxygen-plasma treatment and increase for the nitrogen-plasma (Wang, Lu et al. 2006). The pressure used in our work (4mBar) was higher than the ones used by Wang et al. (0.08 mbar – 0.4 mbar). Another parameter with influence on the BC contact angle is the time of operation, 30 min in the current work. According to previous works (Chan, Ko et al. 1996; Bhat and Upadhyay 2002) a short time (1–3 min) treatment in a nitrogen atmosphere result in more hydrophilic surfaces. In contrast, longer treatments

(i.e. > 3 min) decrease the surface hydrophilicity. Thus, the effect of plasma on the surface hydrophilicity is not straightforward; composition, pressure and time influences in a complex way the final effect. It is not in the scope of this study to analyse this complex function, which demonstrates the versatility of the technique.

In the modification of polymer materials with low-pressure plasmas, various components such as electrons, ions, radicals, as well as UV radiation are involved and interact with the exposed surfaces (Oehr 2003). When high energetic particles of the plasma impact the material surface, chemical bonds are broken while new ones form, thus the chemical environment may change. XPS analysis was employed to analyse the modifications taking place during the plasma treatment. The relative atomic concentration of O, C and N on the BC and BCP surface is shown in table 3.1.

Table 3.1 BC and BCP elemental composition analysed by XPS

Surface Modification	Autoclaved (Y/N)	Oxygen (%)	Carbon (%)	Nitrogen (%)	O/C	N/C
BC	N	45.20	54.79	0.01	0.82	0.0001
BCP	N	38.31	55.79	5.90	0.69	0.1057
	Y	36.66	59.75	3.59	0.61	0.0600

An increase in the concentration of surface nitrogen was provided by plasma environment, as expected. Figure 3.2 shows the XPS survey spectra, highlighting the N 1s peak on BCP. Sterilization at 121 ºC removes nitrogen to some extent; on the other hand, the surface composition of BCP is stable at room temperature. Indeed, after 180 days, the concentration of nitrogen, carbon and oxygen did not show any significant modification (Table 3.2).

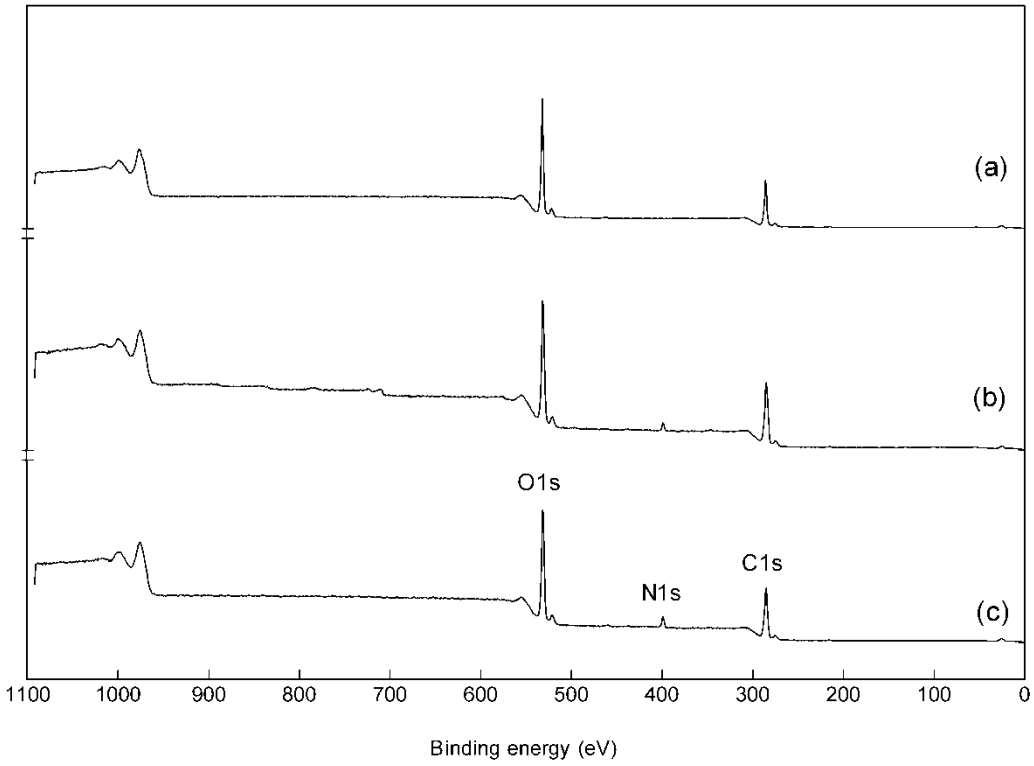

Figure 3.2 XPS survey spectra of: (a) BC; (b) autoclaved BCP and (c) BCP.

Table 3.2 BCP ageing

Surface Modification	Days after treatment	Oxygen (%)	Carbon (%)	Nitrogen (%)	O/C
BCP	15	38.31	55.79	5.90	0.68
	50	38.88	55.83	5.29	0.69
	180	37.81	56.52	5.67	0.66

Additional insight into the surface chemistry can be obtained through deconvolution of the XPS spectra. The C 1s, O 1s and N 1s peaks of BC and BCP are shown in figure 3.3 and table 3.3. The binding energy of C 1s and its spectra deconvolution are well documented. There is a general agreement on the assignment of components C1, C2, C3 and C4 of C 1s peak in wood-derived material (Gray 1978; Dorris and Gray 1978a; Dorris and Gray 1978b;

Mjoberg 1981; Takeyama and Gray 1982; Hon 1984). C1 corresponds to carbon only linked to hydrogen or carbon (-C-H, -C-C); C2 is assigned to carbon linked to a single oxygen (-C-O), whereas C3 binds two non-carbonyl oxygen O-C-O, or a single carbonyl oxygen (-C=0) and finally C4 represents carbon atoms linked to a carbonyl and a non-carbonyl oxygen (O–C=O) (Hua, Kaliaguine et al. 1993).

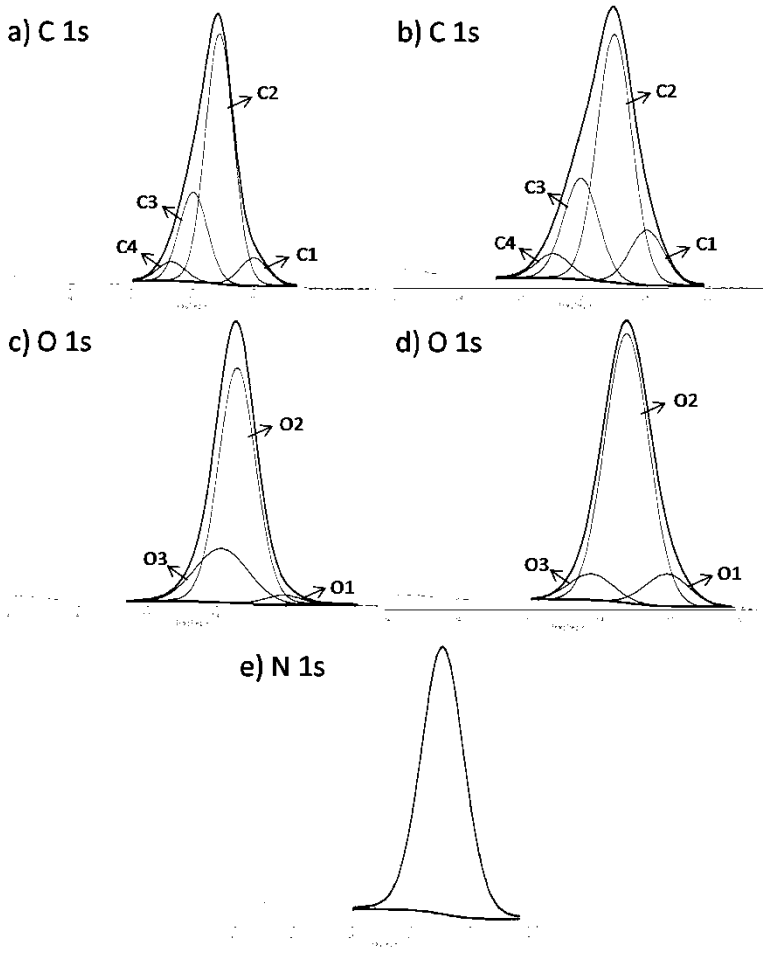

Figure 3.3 Deconvolution of the carbon peak for (a) BC and (b) BCP, and oxygen peak for (c) BC and (d) BCP (e) nitrogen peak for BCP.

Pure cellulose is a homopolysaccharide composed of β-D-glucopyranose units. Each monosaccharide unit contains five carbon atoms linked to one of oxygen and another carbon linked to two oxygen atoms. Thus, one expects a curve-resolved XPS C 1s signal to consist of only two peaks (C2

and C3). The carbon composition, C1/C2/C3/C4 for the pure cellulose is expected to be [0:83:17:0] (Carlsson and Strom 1991). However, the carbon composition determined using filter paper, as given by XPS measurements in a work developed by Carlsson and Strom (1991), was found to be [8:72:17:3]. According to these authors, the appearance of the two peaks C1 and C4 may be due to either a contamination of the filter paper and/or to a chemical change in the cellulose structure. Likewise, the XPS characterization of bacterial cellulose, by Li et al. (2009), showed that the C (1s) spectra presented three peaks, at 285 eV (C-C), 286.6 eV (C-O, C-OH) and 288.3 eV (O-C-O, C=O). In the present work, BC presented 4 carbon peaks corresponding to C1, C2, C3 and C4. The C1 (C-C) peak should be representative of ubiquitous contamination of cellulose by carbon and oxygen in air-exposed surfaces (Johansson and Campbell 2004). The C4 peak could be attributed to C1 core level of carbon atoms in carboxyl groups (O-C=O) (Sapieha, Verreault et al. 1990; Belgacem, Czeremuszkin et al. 1995).

The O 1s peak in the BC and BCP samples corresponds mainly to two forms of oxygen: O2 oxygen in OH groups of cellulose, O3 oxygen in C-O-C and O-C-O groups; the O1 is the most intense component peak, representing 73.45% and 82.17% of the O 1s total areas in the untreated and treated celluloses, respectively. The O1 peak is related to amide groups $–CONH_2$ (Cagniant, Magri et al. 2002).

Nitrogen plasma induces the incorporation of various chemical functionalities onto the polymer surface. On exposure to this kind of treatment, the incorporation of N-containing functional groups - such as amine, imine, amide, nitrile - on different materials has been described, whose distribution and density can be tuned with the plasma parameters, and depend also on ageing processes (Gancarz, Pozniak et al. 2000; Salerno, Piscioneri et al. 2009). Through nitrogen plasma treatment N-groups were incorporated at the BC surface. The component N 1s was decomposed in only one peak at a binding energy of 400.39 eV. According to the literature (Jansen and van Bekkum 1994; Cagniant, Magri et al. 2002), this binding energy (400.2 ± 0.10

eV) is assigned to amides, lactams and nitriles.

Table 3.3 Concentration of different functional groups on BC and BCP

Bacterial Cellulose		Peaks (%)	Binding Energy (eV)	Assignment
BC	**C1s (1)**	7.22	285.0	Non-functionalised carbon (C-C; C-H)
	C1s (2)	64.56	286.67	Carbon linked to oxygen by a simple bound
	C1s (3)	23.15	287.96	Carbon linked to two oxygen atoms by simple bounds (O-C-O); carbon linked to one oxygen atom by double bound (-C=O); amide ($CO-NH_2$)
	C1s (4)	5.05	289.04	Carbon in –COOR (carboxylic acids, esters, lactones, anhydrides)
	O1s (1)	2.96	531.12	Amide groups $–CONH_2$
	O1s (2)	73.45	533.12	-OH groups of cellulose
	O1s (3)	23.58	533.79	-COOH
BCP	**C1s (1)**	12.79	285.0	Non-functionalised carbon (C-C; C-H)
	C1s (2)	57.75	286.56	Carbon linked to oxygen by a simple bound and carbon linked to nitrogen in nitrile (C-N)
	C1s (3)	23.66	288.17	Carbon linked to two oxygen atoms by simple bounds (O-C-O); carbon linked to one oxygen atom by double bound (-C=O); amide ($CO-NH_2$)
	C1s (4)	5.78	289.52	Carbon in –COOR (carboxylic acids, esters, lactones, anhydrides)
	O1s (1)	9.82	531.21	Amide groups $–CONH_2$
	O1s (2)	82.17	532.93	-OH groups of cellulose

O1s (3)	8.01	534.46	-COOH

The O/C atomic ratio of BC was found to be 0.82 (table 3.1), a value in agreement with the theoretical one in pure cellulose (0.83) (Topalovic, Nierstrasz et al. 2007). After the plasma treatment, the O/C atomic ratio changed to 0.69 and 0.61, for the non-sterilized and sterilized BCP, respectively. These values are close to those - 0.55 and 0.62 - reported by Topalovic (2007) and Li (2009) (Topalovic, Nierstrasz et al. 2007; Li, Wan et al. 2009).

Changes in hydrophilicity induced by the implantation of polar functional groups can be time-dependent, an effect often called “ageing”, reported by many researchers on modified polymer surfaces. The nitrogen plasma modification of BCP membranes was examined along time by XPS, in order to access the stability of the nitrogen groups incorporated on the surface. Chain migration in the surface region can result in gradual deterioration of the surface properties. Our results showed that the functional groups present on BCP surface are stable at room temperature, up to 180 days. The long-term stability of a modified polymer surface is important whenever the material is not stored in a controlled environment or coated immediately after treatment. Several factors have been reported to influence the stability of modified surfaces, including contamination, the chemical structure of the original polymer, electrical properties, the degree of plasma modification (operational conditions), the plasma gas, the storage environment, all may contribute to the overall decay phenomenon (Gerenser 1993; Bhat and Upadhyay 2002).

In the work developed by Bhat and Upadhyay (2002), the effect of storage time on the surface energy of unoriented polypropylene films (UPP) treated with nitrogen plasma was analysed over a period of 2 months. UPP films treated in nitrogen plasma for 3 min showed a gradual reduction of the surface energy along with the storage time, whereas films treated for 10 min were stable.

According to the authors, the re-orientation of mobile groups is responsible for the observed change in surface energy. For the shorter treatment time, hydrophilic groups are incorporated on the polymer surface, and as a result, surface energy is improved only for freshly treated samples. However, for the samples stored for 2 months, hydrophilic groups reorients toward each other and also toward the interior. Because of this, hydrophilic groups do not face the air–liquid interface and therefore the contact angle increases and surface energy decreases. For a longer treatment time (10 min), the crosslinking reactions avoid the mobile group to reorient easily, such that the surface energy remains constant (Bhat and Upadhyay 2002). In the current case, since BC was treated for 30 min with nitrogen plasma, a crosslinked surface, leading to a permanent binding of N atom to the polymer chain, may thus be responsible for the observed stability.

Sterilizability is a mandatory requirement for biomedical materials, which must contact cells or tissues. Among the various methods of sterilization, the most frequently applied is hot vapor sterilization (121 ^{o}C, 21 min). This method is preferred as long as the materials are stable (Oehr 2003). Once bacterial cellulose is a thermally stable material, we evaluated whether the functional groups formed by plasma treatment remains stable after autoclaving. Our results showed a decrease in N_2 content (5.90% to 3.59%) after the process. However, even with the decrease of nitrogen after the sterilization, the functional groups present on the surface were able to enhance the cell affinity for BC.

It has been demonstrated, using several materials (Vidaurre, Achete et al. 2001; Yang, Bei et al. 2002; Lucchesi, Ferreira et al. 2008), that plasma may increase the surface roughness. The SEM observations reveal that plasma produces morphological changes on BC. Figure 3.4 shows SEM images of BC and BCP, autoclaved and non-autoclaved. While BC presents relatively low porosity and a tight interfibre contact, the plasma treatment seems to disrupt the fibres to some extent, leading to a more porous and rough material. These changes in the surface topography are mostly caused by chemical erosion and

physical erosion by atoms and ions in the plasma (Vesel, Junkar et al. 2008). However, unlike reported by Yang et al. (2002), the increased roughness does not lead in this case to the enhancement of the hydrophilicity of the plasma treated samples, as discussed previously. Furthermore, the SEM images show the typical micro-channels pattern of BC, which maintained the 3D network structure after plasma treatment. Apparently, autoclaving increases even more the porosity of the scaffold (Fig 3.4). The plasma treatment and autoclaving resulted in BC membranes with larger inter-fiber porosity, which is likely to favour the permeability of nutrients and cell communication and thus representing a promising method for the development of BC scaffolds for tissue engineering. Actually, the change in roughness and porosity has been shown to play a significant effect on the protein and cell attachment, while oxygen as well as nitrogen containing plasma has been shown to increase endothelia cell attachment (Vesel, Junkar et al. 2008).

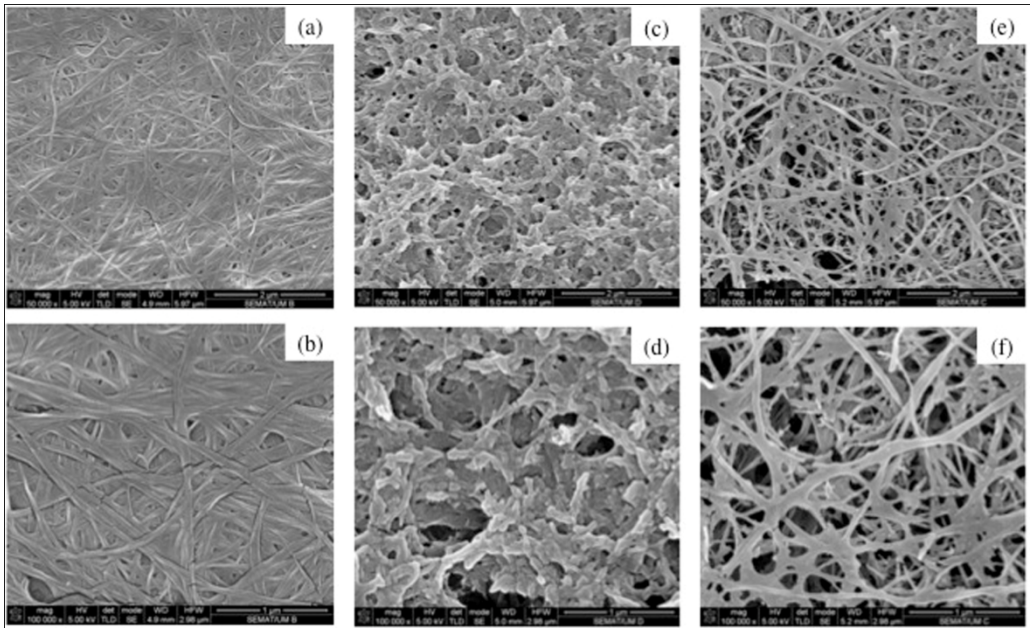

Figure 3.4 SEM micrographs of bacterial cellulose. BC (a, b); BCP (c, d) and autoclaved BCP (e, f).

Biocompatibility is not an inherent property of a material, but results from complex interactions between an implant and the surrounding tissues. Any polymer used in biomedical application should be biocompatible, which requires, among other properties, a low friction coefficient, appropriate surface topography, chemistry and hydrophilicity (Wei, Yoshinari et al. 2007; Gomathi, Sureshkumar et al. 2008). It is known that BC is a very hydrophilic polymer; however, cell-material interactions are not only influenced by a defined balance of hydrophilicity/hydrophobicity, but also by the presence of special functional groups (Klee, Villari et al. 1994). In tissue engineering, cell adsorption is critical, because adhesion occurs before other events like cell spreading, migration and differentiation (Wan, Yang et al. 2003). In this context, BC may be improved as to induce a better cell adhesion and even better biocompatibility. The nitrogen-plasma treatment was chosen and proved to effectively enhance cell affinity through functional nitrogen groups grafted on the surface material. The MTS results (Fig 3.5) showed that the modification by nitrogen plasma efficiently improve the adhesion of N1E-115 and HMEC-1 cells, by 2 fold in the case of HMEC and by 25% in the case of neuroblasts (according to the viable cells detected 2h after cell seeding). Not only the cells adhere in higher number, proliferation is also more exuberant on BCP. However, in the case of the 3T3 fibroblasts, the treatment showed no effect in the timeframe analyzed (2-48 h). Thus, different cell display a different behaviour in contact with modified biopolymers.

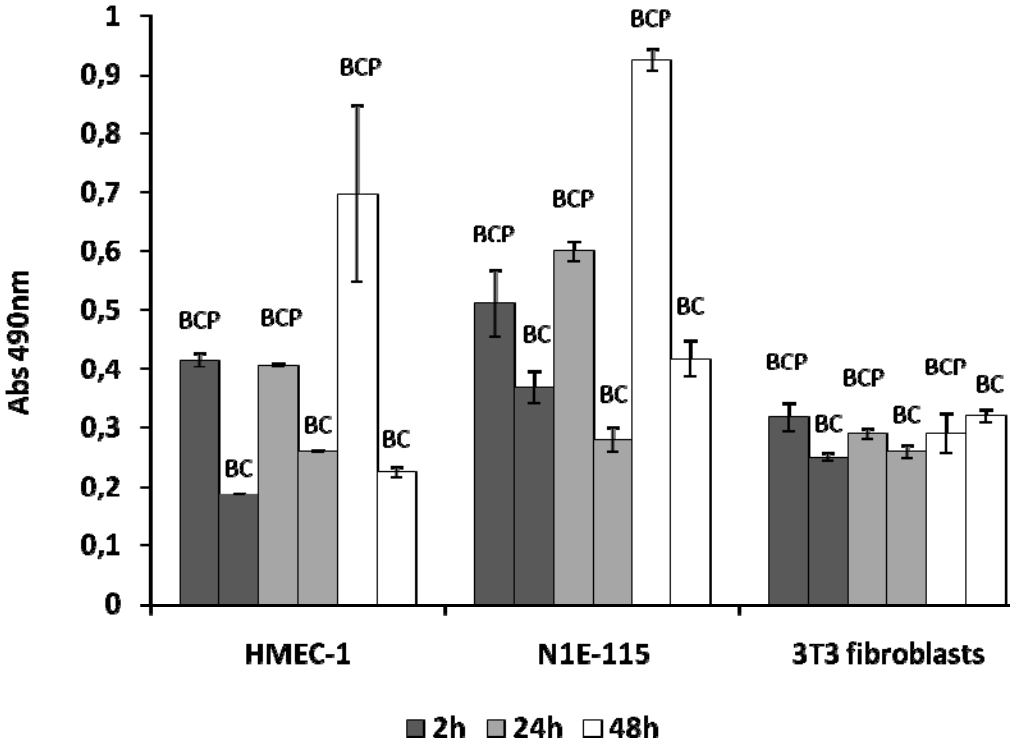

Figure 3.5 MTS assays of HMEC-1, N1E-115 and 3T3 fibroblast cultured on BC and BCP. The MTS assay was developed at 2, 24 and 48 hours after cells addition. Results are expressed in terms of absorbance.

3.5 Conclusions

Plasma surface modification is an effective and economical surface treatment technique, drawing great interest in biomedical engineering. The nitrogen plasma treatment used in this work was able to increase the concentration of functional groups on BC surface in a very stable way along time, and was also capable to improve the adhesion of endothelial and neuroblast cells to the material. Therefore, the surface modification leads to a better cell affinity with BC, probably contributing for a better biocompatibility in vivo. It must be remarked that the plasma treatment improves significantly the porosity of the material. This is a very important result, since the relatively low porosity of BC is a main drawback in the development of tissue engineering applications, because it is the reason for the poor cell penetration. Thus, forthcoming work will address the characterization of the mechanical properties

of the treated BC as well as the analysis of the cell migration through the material, and viability of the cells inside the plasma treated BC.

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4. Bacterial cellulose: long-term biocompatibility studies

4.1 Abstract

The bacterial cellulose (BC) secreted by *Gluconacetobacter xylinus* is a network of pure cellulose nanofibers, which has high crystallinity, wettability and mechanical strength. These characteristics make BC an excellent material for tissue engineering constructs, noteworthy for artificial vascular grafts.In this work, the *in vivo* biocompatibility of BC membranes produced by two *G. xylinus* strains was analyzed through histological analysis of long-term sub-cutaneous implants in the mice. The BC implants caused a mild and benign inflammatory reaction that decreased along time and did not elicit a foreign body reaction. A tendency to calcify over time, which may be related to the porosity of the BC implants, was observed, especially among the less porous BC-1 implants. In addition, the potential toxicity of BC nanofibers – obtained by chemical-mechanical treatment of BC membranes - subcutaneously implanted in mice was analysed through bone marrow flow cytometry, blood and histological analyses. After 2 and 4 months post implantation, the nanofibers implants were found to accumulate cytoplasmically, in subcutaneous foamy macrophages aggregates. Moreover, no differences were observed between the controls and implanted animals in thymocyte populations and in B lymphocyte precursors and myeloid cells in the bone marrow.

4.2 Introduction

Natural and synthetic polymers are used in the field of biomedical materials and tissue engineering in a variety of applications, including among others drug delivery, novel vascular grafts or scaffolds for *in vitro* and *in vivo* tissue engineering (Czaja, Young et al. 2007). These polymers are available in a variety of configurations, including fibers, porous sponges and tubular structures (Kim and Mooney 1998). Tissue engineering approaches typically employ scaffolds made of three-dimensional mimetics of the extracellular matrix (ECMs) to engineer new natural tissues from isolated cells. The scaffolds can be design as macroporous synthetic ECMs, which can regulate the organization of cells seeded into the matrix and its subsequent proliferation to form new tissues. Also, polymeric nanofiber matrices are one of the most promising ECM-mimetic biomaterials because their physical structure is similar to the fibrous proteins in native ECM. However, nanomaterials have unusual properties not found in the bulk material, and this is an important issue because nano-scale and high aspect ratio gives rise to different biological effects compared to micro- and macro-materials (Ma, Kotaki et al. 2005; Koyama, Endo et al. 2006; Barnes, Elsaesser et al. 2008; Moreira, Silva et al. 2009).

Cellulose is a naturally occurring linear homopolymer of glucose, the most widespread polymer in nature (Muller, Muller et al. 2006). Cellulose-based materials, such as oxidized cellulose and regenerated cellulose hydrogels are mainly used in wound healing, as hemostatic agents, hemodialysis membranes and drug-releasing scaffolds (Doheny, Jervis et al. 1999; Helenius, Backdahl et al. 2006; Czaja, Young et al. 2007). Furthermore, several studies reported the applicability of cellulose for culturing cells (hepatocyte, chondrocyte, stem cells) and implantation (bone and cartilage development)(LaIuppa, McAdams et al. 1997; Martson, Viljanto et al. 1998; Entcheva, Bien et al. 2004; Muller, Muller et al. 2006; Pulkkinen, Tiitu et al. 2006).

The bacterial cellulose (BC) secreted by *Gluconacetobacter xylinus* is chemically identical to plant cellulose but different regarding the macromolecular properties and structure (Jonas and Farah 1998; Brown and Saxena 2000;

Klemm, Schumann et al. 2001; Klemm, Heublein et al. 2005). The cellulose secreted by these bacteria is a pure nanofiber network, which has high crystalinity, wettability, mechanical strength, *in situ* moldability, permeability for liquids and gases. Furthermore, it is simple to produce (Vandamme, De Baets et al. 1998; Nakagaito, Iwamoto et al. 2005). These characteristics make BC an excellent material for tissue engineering constructs. Indeed, its potential application as skin substitute for temporary covering of wounds and ulcers, dental implants, scaffold for tissue engineering of cartilage, nerves and blood vessels has been investigated (Fontana, Desouza et al. 1990; Jonas and Farah 1998; Klemm, Schumann et al. 2001; Backdahl, Helenius et al. 2006). Furthermore, BC nanofibers seem to be a good material for biomedical applications since its toxicity has already been evaluated in our previous work, using *in vitro* assays (Moreira, Silva et al. 2009), showing good biocompatibility and no evidence of genotoxicity.

In spite of cellulose-based materials being generally considered biocompatible, showing only a negligible foreign body and inflammatory response *in vivo* (Entcheva, Bien et al. 2004), several parameters must be evaluated as to assess the biocompatibility of an implanted material. Among them, the type and degree of inflammatory and immune response, disintegration, resistance and longevity of the implants in the host tissue have to be taken in consideration (Linde, Alberius et al. 1993; Mendes, Rahal et al. 2009). In this work, the biocompatibility of two different types of BC grafts was analyzed in a long-term approach. Moreover, the biocompatibility of BC nanofibers subcutaneously implanted in mice was also evaluated, including possible effects in lymphopoiesis.

4.3 Material and Methods

4.3.1 Production of bacterial cellulose

The pellicles of BC were produced by the *Gluconacetobacter xylinus* ATCC 53582 (BC-1) and ATCC 10245 (BC-2) strains, cultured in Hestrin-Schramm medium (Hestrin and Schramm 1954), into 96-wells polystyrene plates (250µl per well), for 4 and 7 days, respectively, at 30 ºC in static culture. The membranes

were purified using 2% sodium dodecyl sulfate (SDS) overnight, washed with distilled water until the complete removal of SDS and immersed in a 4% NaOH solution, shaking for 90 min at 60 ^{o}C. After being neutralized, the pellicles were autoclaved in Phosphate Buffered Saline (PBS) and stored at 4 ºC. The BC nanofibers were produced according to Moreira et al. (2009).

4.3.2 Scanning electron microscopy

The BC samples were sputter-coated with gold and examined by scanning electron microscopy (SEM). The analyses were performed on a scanning electron microscope (LEICA S 360). CryoSEM was performed using a microscopy Model Gatan Alto 2500. Samples were fronzen in liquid nitrogen, cut to expose the BC-cells interface, and observed at -150 ºC.

4.3.3 Animals

The *in vivo* biocompatibility studies were performed using male BALB/c mice (8 weeks old) purchased from Charles River (Barcelona, Spain). The animals were kept at the Abel Salazar Institute for Biomedical Sciences of the University of Porto (ICBAS-UP) animal facilities during the experiments. All procedures involving the mice were performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Scientific Purposes (ETS 123) and 86/609/EEC Directive and Portuguese Rules (DL 129/92).

4.3.4 Subcutaneous Implantation

The BC implants were surgically implanted subcutaneously, without fixation, in the back of the mice with each mouse receiving two implants (BC-1 n=48; BC-2 n=80). The mice were anesthetized by an intramuscular injection of a ketamine (Imalgene 1000, Material) and xylazine (Rompun 2%, Bayer Healthcare)

mixture, at a 50 and 10 mg/kg dose, respectively. The hair was removed and two small incisions were made in the skin as bilateral subcutaneous pockets along the backbone where the BC discs were implanted. Finally, the incision pockets were closed with stitches. The control animals received no implants. At least two animals were used for each post-implantation period analyzed (1 week, 1, 3, 5, 7 and 12 months). The aspect of the wound and the presence of edema were evaluated before removing the implants. The implants were removed with the surrounding tissue to prevent damage to the tissue-implant interface, and immersed in formaldehyde for later histological evaluation.

The BC nanofibers were injected in eighteen animals, allocated to in two groups (2 and 4 months post-implantation). Each animal received a 300 µl injection of nanofibers solution (1 mg/ml) on each side of the back, and for the control 300 µl of physiological saline was used. After 2 and 4 months post-implantation, the animals were sacrificed. Blood was sampled by cardiac puncture for blood analysis. Skin surrounding the injection sites and internal organs (liver, spleen, small intestine and mesenteric lymph nodes) were collected for histological analysis.

For flow cytometry analyses, bone marrow cells from femurs and the thymus were collected, washed and ressuspended in PBS, supplemented with 1% BSA and 10 mM of sodium azide. Flow cytometric analysis was performed in a FACScan with the CellQuest software (BD Biosciences), using the following antibodies: FITC-conjugated rat anti-mouse IgM (Pharmingen), FITC-conjugated rat anti-mouse Ly-6E and Ly-6C (Pharmingen), FITC-conjugated rat anti-mouse CD4 (Pharmingen), PE-conjugated rat anti-mouse CD8a (Pharmingen), PE-conjugated anti-mouse CD45R/B220 (Pharmingen), PE-conjugated anti-mouse CD11b (eBioscience). Dead cells were gated out through propidium iodide incorporation.

4.3.5 Histological Analysis

All samples were fixed in 10% neutral buffered formalin for 24 h and paraffin embedded. 4-µm thick sections were used for hematoxylin and eosin (H&E) staining. Slides were examined under a light microscope (Nikon E600); measurements and photographs were obtained with a digital camera (Nikon DS-5M).

4.4 Results

4.4.1 BC morphology

The SEM images of the BC membranes produced by the two strains used in this work (Fig 4.1) exhibit structural differences. BC-1 has a more compact network while BC-2 has a highly porous structure. In addition, the BC-1 membranes obtained in the static culture present, as previously described (Helenius, Backdahl et al. 2006), a more compact surface on the BC-air interface. The BC structure seemed to influence cell invasion and the implant's behavior along time. The Cryosem results shows the compact a porous side of BC-1 membranes, and as can be seen in Fig 4.1 e) and f) the compact side presents aggregated fibrilar structure, while in the porous side, fibers are more dispersed, and the structure have a higher porosity.

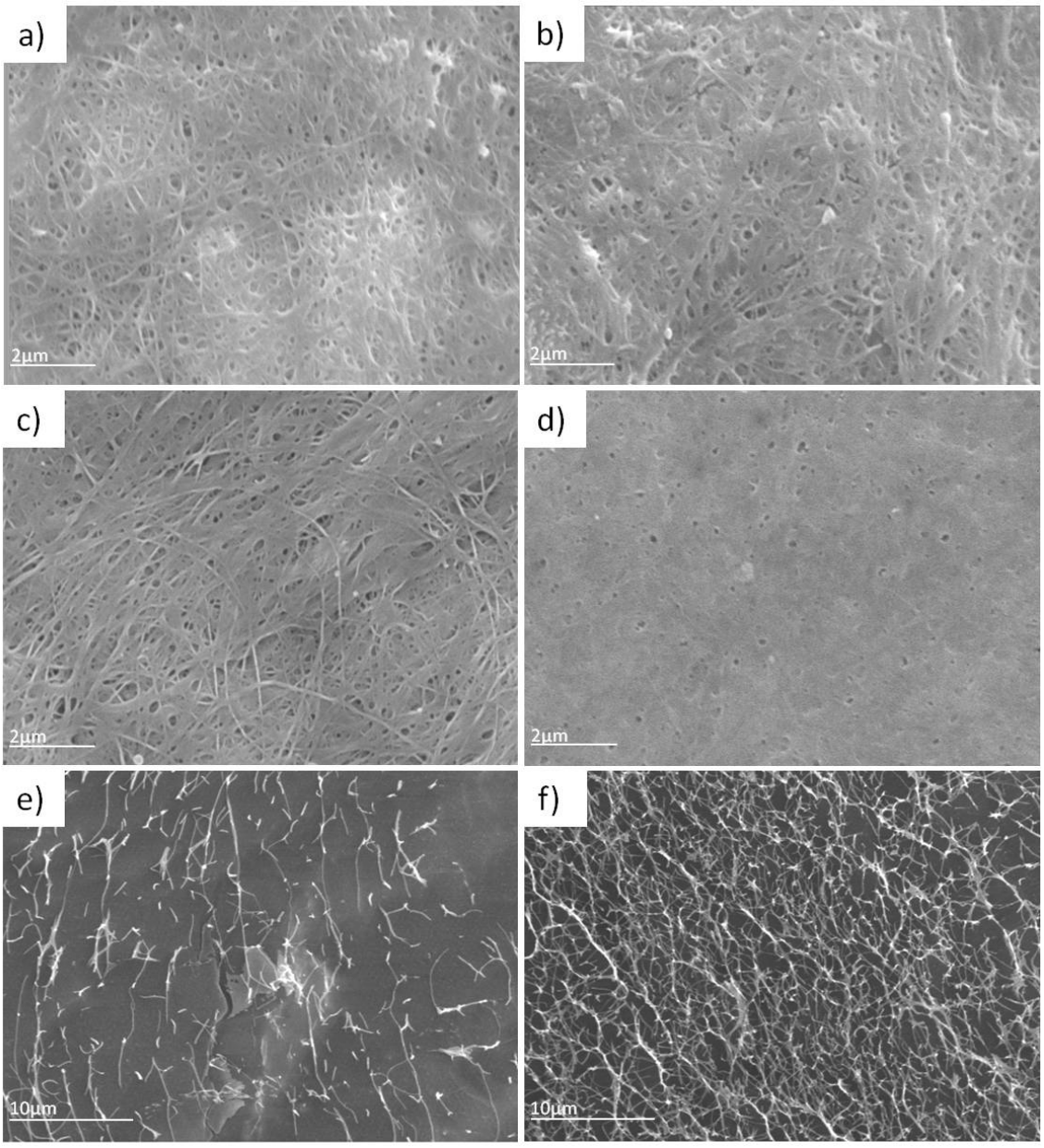

Figure 4.1 SEM micrographs of BC-1 and BC-2. a) BC-2 porous side; b) BC-2 compact side; c) BC-1 porous side; d) BC-1 compact side; and CryoSEM of BC-1 e) porous side and f) compact side.

4.4.2 Bacterial cellulose biocompatibility

On gross examination, the BC implants maintained their shape, but internal fissures lined with migrating mesenchymal cells were evident histologically. No clinical signs of inflammation were present at the incision sites. Cell ingrowth was consistently more extensive on the BC-1 porous side (Fig. 4.2), where cells

presented a spindle-shaped form. Integration with the host tissue was occasionally observed over time, with both materials evaluated; the tissue-implant interface being multifocally obscured by incoming cells (Fig. 4.2). While with BC-1 cellular penetration was more intense through the porous side of the implants, in the case of the BC-2 cells penetrated deeply through both sides of the implants.

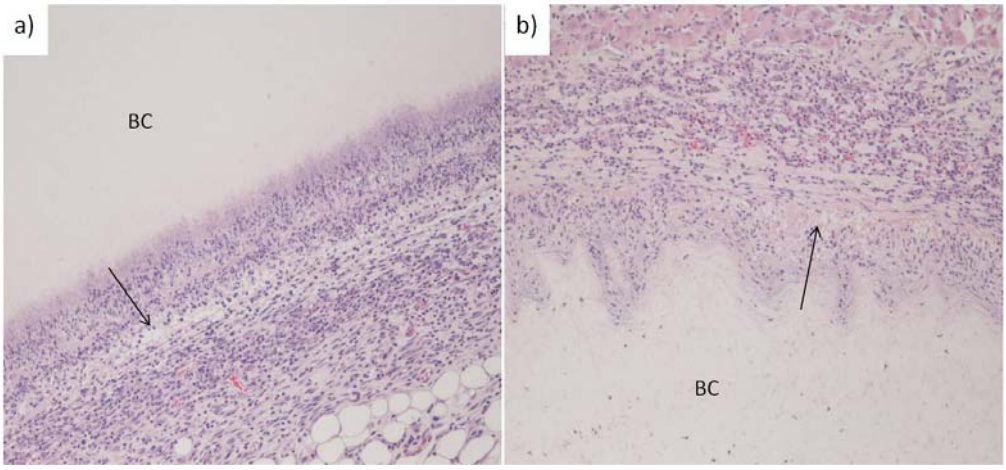

Figure 4.2 Histological images of BC-1, 1 week post-implantation, showing: a) BC-1 compact side; b) BC-1 porous side; and the different pattern of cell infiltration (×100). Arrow head shows the approximate surface between the implant and the connective tissue.

BC did not elicit a foreign body reaction, and only a thin fibrous layer formed. The membrane thickness showed differences between the two materials, ranging from 4 μm to 10 μm to BC-2 and 5 μm to 60 μm to BC-1. A mild, acute inflammation characterized by moderate edema and increased numbers of neutrophils and less macrophages inside and around the implants was observed initially. From 4 weeks onwards, the cell response progressively evolved towards chronicity, with reduced inflammatory cells in and around the implants and a predominance of macrophages over neutrophils. Fibroblasts, endothelial cells and rare adipocytes (collectively referred to as mesenchymal cells on Table 4.1) invaded the implants. After 3 months, the macrophages, fibroblasts and endothelial cells were predominantly found in the implants. Table 4.1 summarizes the qualitative scores regarding different aspects of the biological reaction to the

implants, along time, including calcification, inflammation, presence of blood vessels, and kind of cells observed. Newly formed blood vessels were observed next to the implants. In a few cases (mostly with BC-2 implants) blood vessels were also present inside the implants, although they didn't reach the middle of the implant.

Table 4.1 Qualitative scores of the biological reaction to the BC sub-cutaneous implants

	Calcification	Inflamation	Blood vessels	Cell type	
				M/N	Ms
BC-1					
1 week	-	++++	-	++++	+
1 month	++	+++	-	++++	+++
3 months	+++	+++	+	++++	++++
5 months	+++	++	+	++++	++++
7 months	++	-	++	++++	++++
12 months	+++	+	++	+++	+++
BC-2					
1 week	-	++++	++	++++	++
1 month	-	++++	++	++++	++++
3 months	+	++	++	++++	++++
5 months	+	+++	+	+++	++++
7 months	++	+	+	+++	+++
12 months	++	-	-	++++	++++

(++++) all the implants present the condition

(-) none of the implants present the condition

M/N: Macrophages/Neutrophils

Ms: Mesenchymal cells

Calcification inside BC was observed in many cases (Table 4.1), as shown in Fig. 4.3 and 4.4. In the case of BC-2 calcification occurred, sporadically, 3 months post-implantation and earlier and consistently 1 month post-implantation in the case of BC-1. The calcification localization differed between the two types of implants: BC-1 implants calcified more heavily in the periphery, whereas the calcification of BC-2 spread through the middle of the implant.

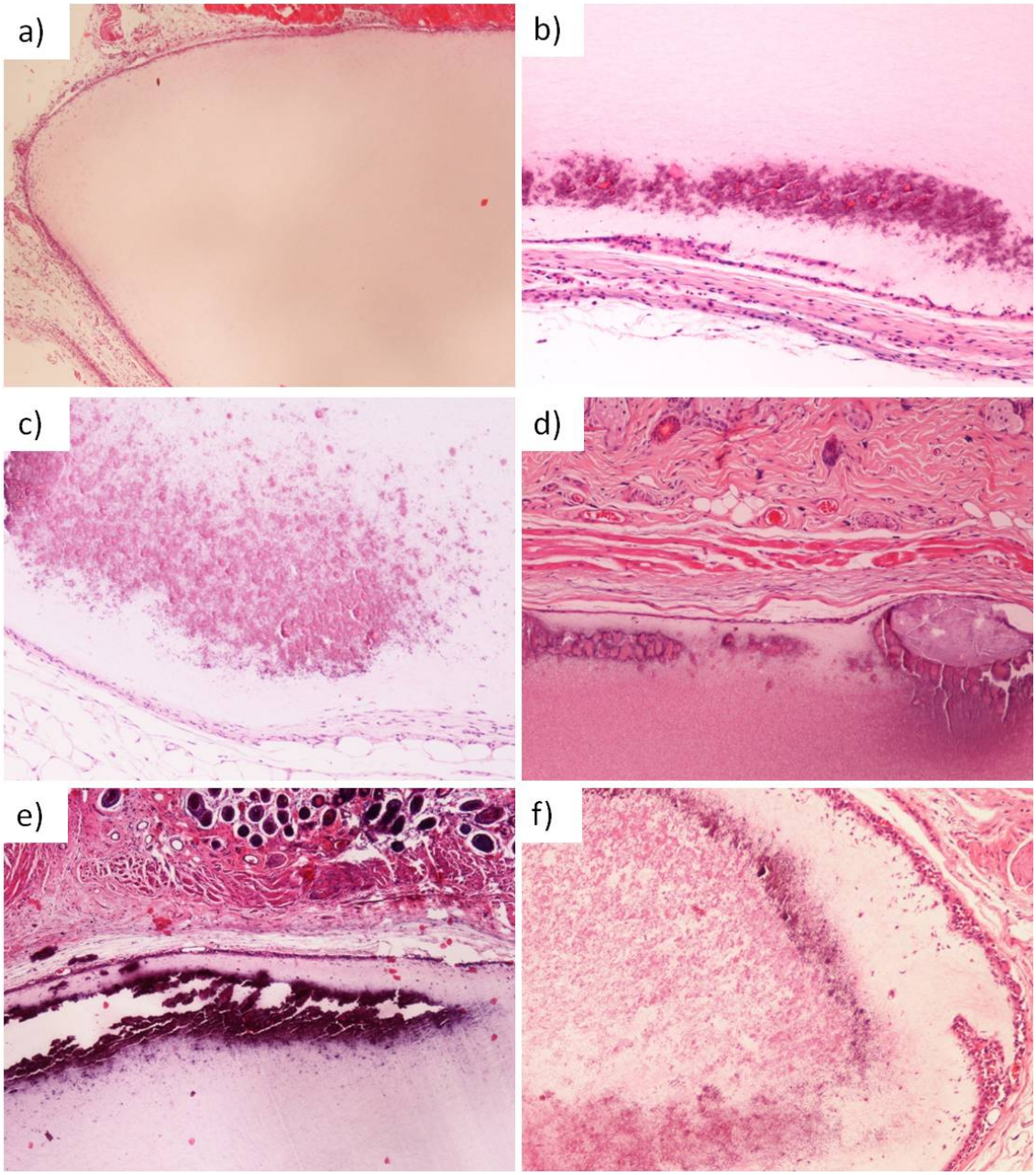

Figure 4.3 BC-1 implants a) 1 week, b) 1 month, c) 3 months, d) 5 months and e) 7 months, f) 12 months post implantation (×100).

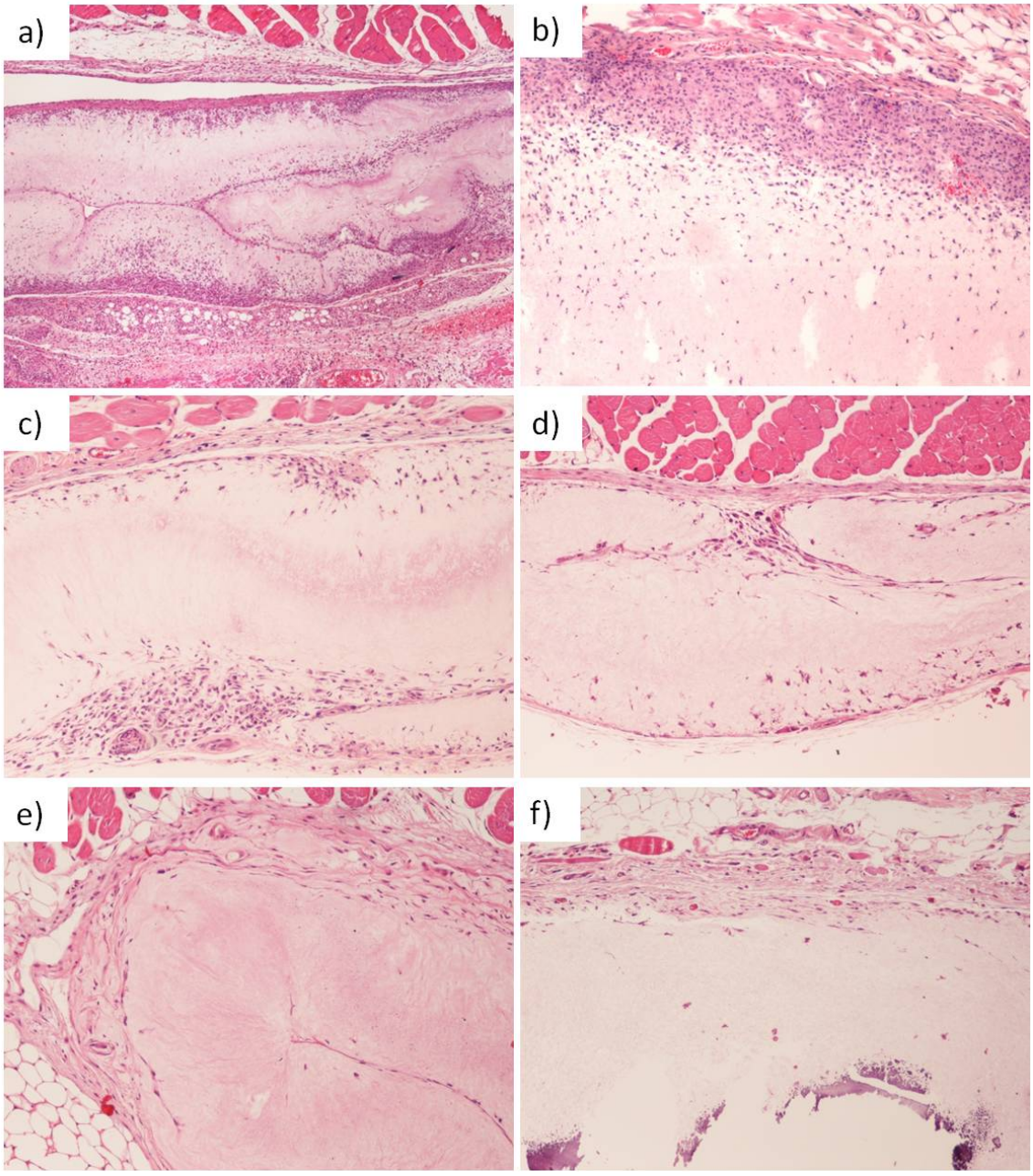

Figure 4.4 BC-2 implants a) 1 week, b) 1 month, c) 3 months, d) 5 months and e) 7 months, f) 12 months post implantation (×100).

4.4.3 Nanofibers

All animals implanted with cellulose nanofibers survived and showed a normal development within the time course of the experiment. There were no significant changes in body weight between the implanted animals and control, nor did any animal show clinical signs of inflammation at the injection sites (data not shown). Histological examination showed a mild, chronic inflammatory process associated with injection sites on nanofibers-exposed mice. At 2 and 4 months post-implantation, nanofibers were present in subcutaneous foamy macrophages aggregates (Fig. 4.5a and 4.5c) as an abundant, lightly basophylic, intracytoplasmic, amorphous material (Fig. 4.5b and 4.5d). In some instances, nanofibers were still present as extracellular deposits surrounded by numerous foamy macrophages engaged in phagocytosis. Small, multifocal, peripheral, lymphoid aggregates and occasional mast cells were also present. Histological analysis of internal organs (small intestine, liver, spleen) showed no differences between implanted and control animals.

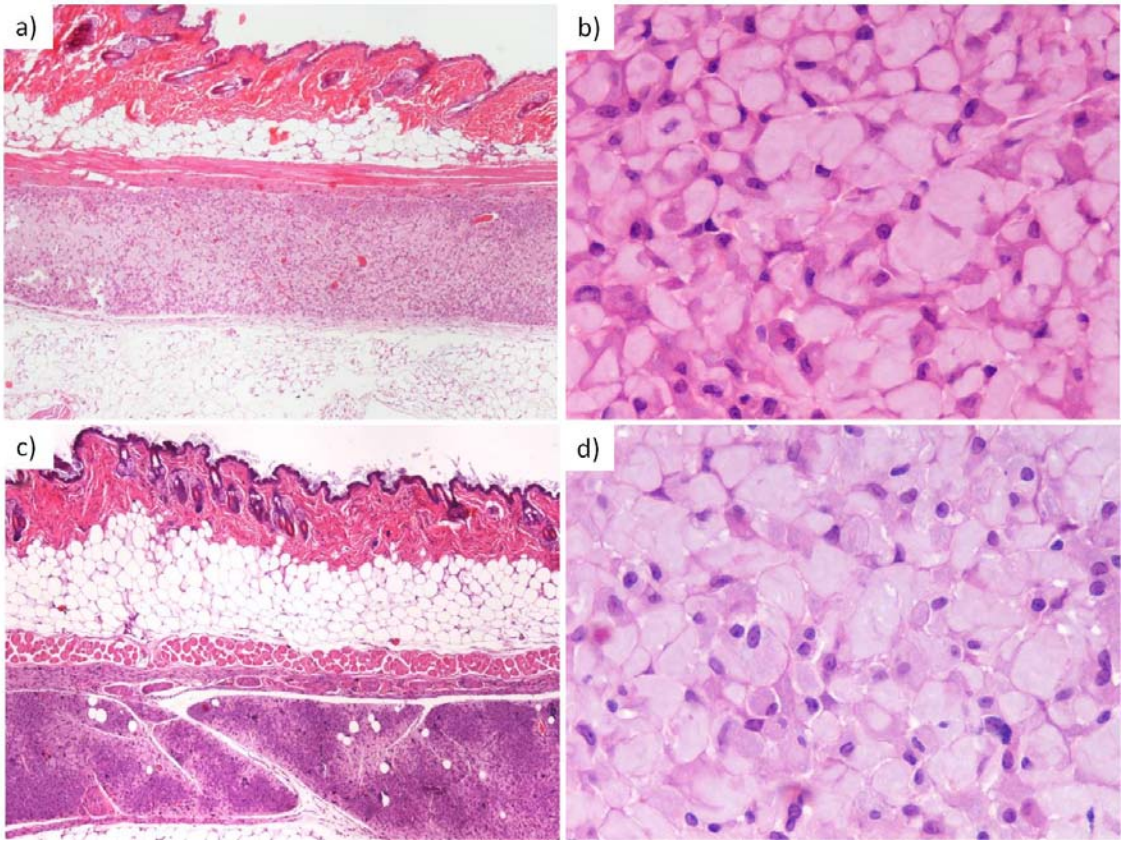

Figure 4.5 Nanofibers implants a) 2 months (×40), b) 2 months (×400), c) 4 months (×40) and d) 4 months (×400), post implantation. a) and c) shows the nanofibers aggregates in the subcutaneous tissue. b) and d) shows the macrophages with intracytoplasmic BC material.

To assess the effect of BC nanofibers in leukocyte hemopoiesis, the proportion of different leukocyte cell populations was analyzed by flow cytometry in the thymus and bone marrow. As shown in Table 4.2, no significant alterations in the proportions of thymic double positive ($CD4^+CD8^+$), or single positive ($CD4^+$ and $CD8^+$) cells were observed in the implanted animals, comparatively to controls, at the time-points analyzed. As also shown in Table 4.2, no significant effect of nanofibers implants was observed in B-cell lineage populations in the bone marrow, as assessed in both pre/pro B ($B220^+IgM^-$), and B immature/mature ($B220^+/IgM^+$) cells. Furthermore, the proportion of bone marrow myeloid/granulocytes cell population ($CD11b^+/GR1^+$) was also not different from that of controls 2 and 4 months upon nanofibers implant. Altogether, these results

indicate that no significant effect in leukocyte hematopoiesis was caused by the implanted BC nanofibers (NFs).

Table 4.2 Cell populations in implanted and control animals

		2 months		4 months	
			BC-NFs		BC-NFs
Cell type	**Marker**	**Control (n=2)**	**(n=6)**	**Control (n=3)**	**(n=5)**
Bone Marrow					
Pre and Pro B cells	$B220^+IgM^-$	25,84 ± 2,57	27,76 ± 1,09	24,75 ± 2,88	30,64 ± 2,49
Immature/mature B cells	$B220^+IgM^+$	17,96 ± 1,25	18,82 ± 0,21	13,37 ± 1,32	14,01 ± 0,60
Granulocyte/myeloid cells	$CD11b^+GR\text{-}1^+$	52,89 ± 2,67	50,22 ± 1,57	46,88 ± 2,84	45,10 ± 3,24
	$CD11b^+GR\text{-}1^-$	5,48 ± 0,01	6,67 ± 1,04	3,43 ± 0,52	3,35 ± 0,35
Thymus					
$CD4^+CD8^+$ double positive thymocytes	$CD4^+CD8^+$	84,47 ± 1,32	85,24 ± 1,13	82,87 ± 0,39	84,77 ± 1,06
Single positive CD4 T cells	$CD4^+CD8^-$	6,94 ± 0,15	6,60 ± 0,65	7,84 ± 0,25	6,66 ± 0,78
Single positive CD8 T cells	$CD4^-CD8^+$	1,74 ± 0,22	1,67 ± 0,10	1,69 ± 0,23	1,77 ± 0,20

Data represent cell percentages (mean ± s.d.). *n* = number of mice

4.5 Discussion

Biocompatibility is one of the main requirements of any biomedical material and can be defined as the ability to remain in contact with living tissue without causing any toxic or allergic side effects (Czaja, Young et al. 2007). BC is generally considered a biocompatible material, although, to our knowledge, only two papers reported the study of the fate of sub-cutaneous BC implants *in vivo*; in these cases, the studies were conducted for relatively short periods (up to 12 weeks). In this work, the fate of implanted BC along much longer periods of time

(up to one year), possible differences associated to the structure (namely porosity) of the material and possible toxicity effects related to BC nanofibres were analyzed.

As expected, the BC implants in the present experiment did not elicit a foreign body reaction. Only a thin capsule was formed over time, its thickness depending on the kind of implant (BC-1 implants elicited a stronger encapsulation than BC-2). The inflammatory reaction caused by the implants was mild and didn't cause any complications. In the first weeks, the cells colonizing the implants were mostly neutrophils and macrophages. However, over time, macrophages became predominant over neutrophils, and fibroblasts and endothelial cells were the main cell types within the implants, although blood vessels were restricted to the implant's periphery. Integration with the host tissue was multifocally present, in areas where incoming cells obscured.

These results are in agreement with Helenius et al. (2002) who implanted BC subcutaneously in mice, for a period of up to 12 weeks. No signs of chronic inflammatory reaction or capsule formation were verified in that case, and the formation of new blood vessels around and inside the implants was observed (Helenius, Backdahl et al. 2006). In another work, Mendes et al. (2009) subcutaneously implanted BC was shown to be nonresorbable and capable of inducing a mild inflammatory response. The authors observed at 60 and 90 days post-surgery no inflammatory infiltrate. The angiogenesis was markedly reduced and the connective tissue surrounding the membrane was mature (Mendes, Rahal et al. 2009).

In this work, the structural differences between BC produced by the different *G. xylinus* strains determined the cell behavior in the implants. In the case of BC-2 membranes, cells were able to migrate into the inner membranes and colonize their full extent. In the case of BC-1, cell migration was conditioned by the tight pores, and by the compact and soft sides, cells being unable to reach the inner portion of the implants. As described by Helenius et al. (2005), our results indicate that the magnitude of cell ingrowth seems to be dependent on the porosity of BC,

cell migration declining where the material is denser (Helenius, Backdahl et al. 2006).

In this study, in contrast to earlier reports referred, BC calcification was, for the first time, observed. This type of calcification may be due to the occurrence of cell death inside the BC structure. Cell death results in an acid environment in the implant, conducive for the mobilization and concentration of calcium. The tentative explanations of biomaterials calcification assign the main cause to dead cells. According to this hypothesis, the accumulation of calcium deposits originates from the cells or tissues that have degenerated or become necrotic (Nomizu, Weeks et al. 1995; Zainuddin, Chirila et al. 2005). However, some authors showed that, even without direct contact with cells, calcification of biomaterials occurs through the formation of a protein–calcium complex layer on the surface of biomaterials, this being the key event in biomaterial calcification (Rosanova, Mischenko et al. 1991; Vasin, Rosanova et al. 1998). Furthermore, Rosanova et al. (1991) suggested that the formation of calcium deposits occurs by the adsorbed protein molecules, which bind Ca^{2+} ions from surrounding media; alternatively, Ca-protein complexes forms at the biomaterial/blood interface and adsorbs onto the surface.

This work provides clues regarding the factors influencing BC calcification. The porosity and time of implantation are factors that seem to influence calcification, considering that the two BCs used presented different calcification patterns (peripheral versus diffuse). Calcification only occurred in the implants where cells were present, preferentially in the interior of the implant, and at different times according to the type of cellulose. The findings suggest that the differences in membrane size and the longer observation period compared with previous studies (ref) may have led to different observations in our study.

4.5.1 Nanofibers

Nanofiber matrices are well suited to tissue engineering: 1) as scaffold that can be fabricated and shaped to fill anatomical defects; 2) its architecture can be designed to provide the mechanical properties necessary to support cell growth,

proliferation, differentiation, and motility; 3) and it can be engineered to provide growth factors, drugs, therapeutics, and genes to stimulate tissue regeneration. An inherent property of nanofibers is that they mimic the extracellular matrices (ECM) of tissues (Ramakrishna, Fujihara et al. 2006) and the different biomacromolecules, which compose the native ECM and their organization. Furthermore, its nanoscale dimension can provide tensile strength and elasticity for the tissues (Ma, Kotaki et al. 2005). Although BC is not expected to be resorbable, some authors show evidence that amorphous may undergo degradation *in vivo*. Thus, the possibility that nanofibers may be released from implanted BC made materials cannot be ruled out, hence the need to study the toxicity of BC nanofibers. Indeed, since the nanomaterials have unusual properties, not found in the bulk material, such as high surface reactivity and ability to cross cell membranes, concerns about their safety and toxicology emerged (Moreira, Silva et al. 2009). The impact of nanostructural features in the interaction of a material with cells and tissues is dependent on the size, chemical composition, surface structure, solubility, shape, and on the supramolecular structural organization (Barnes, Elsaesser et al. 2008; Moreira, Silva et al. 2009). In this context, the toxicity of nanoscale substances has been studied and it is known that carbon nanotubes and asbestos are nanoscaled materials with carcinogenic potential (Speit 2002; Donaldson, Aitken et al. 2006; Poland, Duffin et al. 2008)

Moreira and co-workers in 2009 presented the first evaluation of the potential genotoxicity of BC nanofibers and showed that BC NFs did not present genotoxicity *in vitro*. However, an *in vivo* study is still missing and it is well known that with the *in vitro* systems there is no possibility to evaluate secondary inflammatory effects (Moreira, Silva et al. 2009). Some studies with cellulose fibers described the biological effects of this type of material in animal studies. Cellulose fibers tested *in vivo* showed no adverse health effects when chronically ingested, but when present in the intraperitoneal region can cause mesothelioma in rats, and when inhaled, have the potential to accumulate and induce pathological changes in the lung (Anderson, Owens et al. 1992; Adamis, Tatrai et al. 1997; Cullen, Miller et al. 2002). In this work, most injected nanofibers

remained in macrophage aggregates in the subcutaneous tissue and did not cause any visible abnormalities neither in the host adjacent tissue nor in the abdominal organs. These results are in agreement to studies conducted with carbon nanotubes and carbon nanotubes/polycarbosilane composites, which were implanted in the subcutaneous tissue of rats and showed no acute inflammatory response, such as necrosis. In addition, the carbon nanofibers were internalized by the macrophages and foreign body giant cells, which were found in the implants local (Yokoyama, Sato et al. 2005; Wang, Watari et al. 2007).

Systematic analyses of nanomaterials biocompatibility are essential to the use of these structures in tissue engineering applications. The *in vivo* systemic effects of nanomaterials were studied by some authors, for instance, activated carbon fibers and asbestos implanted subcutaneously in mice were analyzed by Koyama and co-workers (2002) and asbestos-implanted tissue showed a severe inflammatory reaction and formation of abscess-like mass in the implanted tissue along with low values of peripheral blood $CD4^+$ and $CD8^+$ T cells (Koyama, Tanaka et al. 2002). Koyama also showed the relative low toxicity of different types of carbon nanotubes, subcutaneously implanted in mice. The carbon nanotubes gave rise to several characteristic time-dependent changes in $CD4^+$ and $CD8^+$ T-cells values (Koyama, Endo et al. 2006). Furthermore, it was shown that the inhalation of asbestos has adverse effects in leukopoiesis in mice, leading to a depression of the number of bone marrow pluripotent stem cells and marrow granulocyte macrophage progenitors (Boorman, Dean et al. 1984). In our work, the absence of BC nanofibers toxicity *in vivo* was further evidenced by the absence of observed disease features in mice. Flow cytometry analyses did not show any significant effect in leukocyte hematopoiesis caused by the implanted BC nanofibers. No significant alterations in the proportions of thymic double positive ($CD4^+CD8^+$), single positive ($CD4^+$ and $CD8^+$) cells, B-cell lineage populations, and myeloid/granulocytes cell population ($CD11b^+/GR1^+$) were observed in the bone marrow, in the implanted animals comparatively to controls. Therefore, these results indicate that BC nanofibers did not cause a significant inflammatory response and can be considered an innocuous material *in vivo*, suitable for tissue engineering applications.

4.6 Conclusion

The BC is considered a great material to implants with good biocompatibility characteristics. However, our work points to the necessity to further investigation to verify the tendency to BC to calcify in long-term *in vivo* circumstances. Porosity is likely to be the main limitation for a widespread colonization of the material, allowing for proper tissue integration and the production of neo-tissues with excellent mechanical properties. The calcification detected in this work seems to be mainly dependent on the material's porosity and on the exposure period. Although calcification is an undesirable fate for such a biomaterial, it should be remarked that such events may be dependent on the tissue where the biomaterial is to be placed. On the other hand, a proper porosity allowing angiogenesis and adequate nutrients supply to the cells may avoid the calcification processes. The BC nanofibers seem to be an innocuous material in mice subcutaneous tissue, and proved to be an eligible material to production of ECM-mimetic grafts.

4.7 References

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5. Conclusions and perspectives

This work presented two strategies to modify the BC to enhance its biocompatibily: the surface modification with the incorporation of amino groups through nitrogen-plasma treatment, and BC modification through the adsorption of recombinant proteins with different bioactive peptides. Our experimental results indicate that these techniques are efficient in the modification of BC, producing scaffolds capable to select and promote cell adhesion and viability. The modified BC has the potential to be used in neural tissue engineering. The treatment with other gases and the use of different bioactive molecules on the production of recombinant proteins are alternatives to further modify BC and other biomaterials to be use in tissue engineering.

Also, our study provides a long-term approach of BC implants and nanofibers biocompatibility *in vivo,* an important issue to consider when we envisage the use of a nondegradable material for the construct of tissue engineering devices. This work aims to complement the lack of information about the effects of BC implanted for a long time *in vivo*, and also the effect of BC nanofibers implanted in mice. From the BC implants we conclude that porosity is likely to be the main limitation for calcification tendency and a widespread colonization of the material, allowing for proper tissue integration. The BC nanofibers seem to be an innocuous material in mice subcutaneous tissue, and proved to be an eligible material to production of ECM-mimetic grafts.

Our future work in this area will include:

- Mesenchymal stem cells encapsulation and analysis of cell viability, spreading, neurotrophin expression and release in different scaffolds produced with the modified BC combined with other materials.

- Evaluate the effects of the neurotrophins released by the MSCs on the survival and differentiation of other cell types *in vitro*.

- *In vivo* biocompatibility of acellular scaffolds composed of BC modified with nitrogen plasma or with the recombinant proteins on a sciatic nerve injury model in rats.

- *In vivo* biocompatibility of cellular scaffolds using the modified BC with MSCs adhered and releasing neurotrophins on a sciatic nerve injury model in rats.

- Functionalization of BC nanofibers to more closely mimic the ECM and to be used in tissue engineering applications.