Universidade do Minho
Escola de Engenharia

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Synthesis and Characterization of Electrical Conductive Bacterial Cellulose-graft-Polyaniline

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Synthesis and Characterization of Electrical Conductive Bacterial Cellulose-graft-Polyaniline

Dissertação de Mestrado
Mestrado em Micro/Nano Tecnologias

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Doutor Fernando Dourado

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É AUTORIZADA A REPRODUÇÃO PARCIAL DESTA DISSERTAÇÃO APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE;

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II. Abstract

In recent years, conductive polymeric nanomaterials have received considerable attention because of the increasing demand of new technologies for the development of electronic devices, sensors, scaffolds, to be used in the most diverse research areas such as biology, tissue engineering, food industry, etc.

In this context interest in polyaniline (PANI) has grown exponentially, being regarded as one of the most technologically capable electrical conducting polymers, due to its high electrical conductivity, easy synthesis, low cost, and stable electrical conductivity. Bacterial cellulose (BC) nanofibers appear as very promising support material for these conductive additives due to their high strength and stiffness associated to high purity, high porosity, and biocompatibility. The combination of these two materials opens a new field of potential applications for bacterial cellulose.

This project aim was to develop an electrical conductive bacterial cellulose-graft-polyaniline composite by means of the oxidative-radical copolymerization using ammonium persulfate in acidic medium. The grafting conditions were studied by varying grafting parameters: monomer concentration and polymerization time.

Different methods were studied for the producing of the conductive BC-graft-PANI composites: in situ direct polymerization of aniline in BC discs; surface modification of BC; and sulfonation of BC. The electrical conductivity increased from $7.5*10^{-11}$ S/cm to $2.26*10^{-4}$ S/cm by controlling the time of polymerization and the molar Ratio of CB:aniline. Under the assayed experimental conditions, the optimum grafting efficiency was find at a CB:aniline ratio of 1:10 and with a time reaction of 6 hours.

The produced BC-graft-PANI composites were characterized using conductivity assays, scanning electron microscopy (SEM), fourier-transformed infrared spectroscopy (FTIR), thermogravimetric analysis (TGA), differential scanning calorimetry (DSC), and viability assay (MTS test) taking BC as reference.

III. Resumo

Nos últimos anos, os polímeros condutores tem recebido especial atenção devido há crescente necessidade de novas tecnologias para o de envolvimento de dispositivos electrónicos, sensores, suportes porosos, que poderão ser usados nas mais diversas áreas como a biologia, a engenharia de tecidos, a industria alimentar, etc..

Neste contexto, o interesse na polianilina (PANI) tem crescido exponencialmente, sendo considerada um dos polímeros condutores mais promissores, devido à sua elevada condutividade eléctrica, fácil síntese, baixo custo e estabilidade térmica. No que diz respeito às nanofibras de celulose bacteriana, estas surgem como um material de suporte muito interessante para este tipo de polímeros condutores devido às excelentes propriedades mecânicas, associadas á elevada pureza, elevada porosidade, e biocompatibilidade. Com a combinação destes dois materiais abre-se um novo campo de aplicações para a celulose bacteriana.

Este projecto tem como objectivo o desenvolvimento de um material compósito condutor à base de celulose bacteriana e polianilina através da polimerização oxidativa *in situ* da anilina em meio acido e usando persulfato de amónia como agente oxidante. As condições de polimerização foram estudadas fazendo variar os parâmetros de reacção: concentração do monómero e tempo de polimerização.

Para a produção dos compósitos BC/PANI foram estudados diferentes métodos: a polimerização directa *in situ* da anilina na celulose bacteriana em meio ácido; modificação da superfície da celulose bacteriana, através da activação inicial da superfície da CB e posterior polimerização oxidativa; e sulfonação da celulose bacteriana. A condutividade eléctrica aumentou de $7.5*10^{-11}$ S/cm para $2.26*10^{-4}$ S/cm controlando o tempo de polimerização e a razão molar BC:anilina. Para as condições analisadas, os melhores resultados obtidos foram a razão molar CB:anilina de 1:10 e um tempo de reacção de 6 horas.

Os materiais obtidos foram caracterizados através de ensaios de condutividade, microscopia electrónica de varrimento (SEM), espectroscopia de infravermelho (FTIR), analise termogravimétrica (TGA), calorimetria diferencial de varrimento (DSC), e ensaios de viabilidade (teste MTS), usando a celulose bacteriana como referência.

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CHAPTER I. INTRODUCTION

CHAPTER II. STATE OF THE ART

CHAPTER III. SYNTHESIS AND CHARACTERIZATION OF ELECTRICAL CONDUCTIVE BACTERIAL CELLULOSE-GRAFT-POLYANILINE

VII. Abbreviations List

APS ----- Ammonium peroxydisulfate

BC ----- Bacterial Cellulose

DBSA ----- Dodecylbenzenylsulfonic acid

DIOHP ----- Ply(methylmetacrylate)

DSC ----- Differential Scanning Calorimetry

EDTA ----- Ethylenediamine tetraacetic acid

FTIR ----- Fourier transform infrared spectrometer

G. xylinus ----- *Glucoacetobacter xylinus*

HS ----- *Hestrin-Scharamm (Medium)*

MWCNTs ----- *Multiwalled carbon nanotubes*

NMP ----- N-methylpyrrolidinona

PANI ----- Polyaniline

PPy ----- Polypyrrole

p-TSA ----- p-toluenesulfonic acid

SEM ----- Scanning Electron Microscopy

3T3 cells ----- Standard fibroblast cell line

TGA ----- Thermogravimetric analysis

CHAPTER I.

INTRODUCTION

1. Motivation and Aims

Polyaniline (PANI), one of the most common conducting polymers, is prepared by the oxidation of aniline in an acidic aqueous medium. It bears good environmental, thermal and chemical stability, electrical and optical properties, facile redox and pH-switching behavior. It can be rapidly 'switched' with the addition of acids (protonation) and bases (deprotonation).

Polymer–polyaniline (PANI) conductive polymeric composites have been received considerable attention because of their potential applications in electrodes, biosensors, batteries, antistatic coatings, gas sensors, membranes, light emitting diodes and notability in neuronal tissue engineering, robotics and biomedical actuators. The rationale is based on the putative modulatory effect of the electrical stimulation on cell attachment, proliferation, migration and differentiation.

Novel electro-conductive bacterial cellulose (BC) scaffolds could result from the combination of the electrical conductivity, chemical stability and thermal stability of PANI with the unique properties of BC: highly homogeneous structure, high moldability *in situ*, high water-holding capacity, biocompatibility and remarkable strength (Young's modulus of 15-30 GPa).

BC is excreted by *Gluconacetobacter xylinus,* a strict aerobe Gram-negative bacterium. Under static culture, BC is organized as a 3D ultrafine network structure. BC is a naturally nano-structured biopolymer, with fibers having less than 100nm wide and composed of elementary nanofibrils, aggregated in bundles with lateral size of 7-8nm. Further, nano-whiskers can easily be obtained by acid hydrolysis of BC, expanding the potential use of BC. The surface modification of BC whiskers has a wide and underexploited potential. Novel functionalized BC nanofibers with predetermined structures, surface-conductive and piezoelectric properties, may allow to improve the properties of materials currently in the marketplace and to create new markets for materials whose manufacture and processing conditions uniquely meet the targeted properties. Their surface modification could improve their stability and their compatibility with the matrices for the synthesis of bio-based nanocomposites.

Due to BC unique properties a few biomedical applications have already reached the market: BASYC are BC cylindrical tubes used in the reconstructive microsurgery of blood vessels. Biofill is used in second and third degree burns, ulcers and as a temporary artificial skin. Gengiflex allows the recovering of periodontal tissues. Xylos Co. also produces BC-based materials for biomedical applications.

The main focus of this project is to explore the nanotechnological potential of surface-modified BC by covalently graft polyaniline onto the surface of BC, through oxidative-radical copolymerization using ammonium persulfate in acidic medium. The grafting conditions were studied by varying grafting parameters.

The representative BC-graft-PANI composites were characterized using conductivity assays, Scanning Electron Microscopy (SEM), Fourier-transformed infrared spectroscopy (FTIR), Thermogravimetric analysis (TGA), Differential Scanning Calorimetry (DSC), and MTS assay taking BC as reference.

2. Context of the work

The present work was developed within the interests of the FUNCARB (FUNctional CARBohydrates Nanobiotechnology) Research Group, at the Center of Biological Engineering (CEB) and in collaboration with the Department of Physics both from the University of Minho. The FUNCARB group belongs to the Associate Laboratory IBB – Institute for Biotechnology and Bioengineering.

The FunCarb Research Group was established in 2010 and operates in the fields of Biotechnology and Biomedical Engineering. This group supports a multidisciplinary and highly skilled team which works at the interface of biotechnology, biology, pharmaceutics, biomedical engineering and materials science. The main goal of FunCarb is to develope tools and new biomaterials based on carbohydrates such as hydrogels, scaffolds, membranes and nano/microparticles, for biomedical application. The polysaccharides currently used in the group include dextrin, bacterial cellulose, hyaluronic acid, chitosan and mannan. The current research activities of the FunCarb Group contain the following topics: polyssacharide-based biomaterials including bacterial cellulose (to be used for biomedical devices, coatings for food products and composites with new properties), injectable hydrogels for tissue regeneration, and nanogels for the development of delivery systems; bioactive peptides and proteins; enzymatic technology.

3. Conductive Polymers

Until Second World War, all carbon based polymers were rigidly regarded as insulators. The use of conductive polymers in materials' science began in 1977 when Chiang *et al* described the doping of polyacetylene (with conductivity (σ) values before and after doping of 10^{-5} S/cm and 10^{2} S/cm respectively) [1]. Upon realizing that polymers could have electrically conductive properties, researchers pursued new paths with the idea of having lightweight conductors capable of replacing metals in many areas. [2, 3]

Conductive polymers, more commonly known as a "synthetic metals", are a class of functional polymers that have alternating single and double carbon–carbon bonds along the polymeric chains. The series of alternating single and double bonds, which is generated by electron cloud overlap of p-orbitals to form π molecular orbital's, is referred to as a conjugated system [4]. The highly conjugated polymer chain can be assigned reversible chemical, electrochemical and physical properties controlled by a doping/de-doping process [5].

Conductive polymers exhibit the unusual combination of the electrical, electronic, magnetic and optical properties of a metal while retaining the mechanical properties, solubility, processibility, etc., commonly associated with a conventional polymer [6]

There is no singular method for synthesizing conductive polymers (Table 1). Conductive polymers may be synthesized by using any one of the following techniques: chemical polymerization, electrochemical polymerization; photochemical polymerization, metathesis polymerization, concentrated emulsion polymerization, inclusion polymerization, solid-state polymerization, plasma polymerization, pyrolysis and soluble precursor polymer preparation. Among these methods, chemical polymerization is the most useful for preparing large amounts of conductive polymers. Chemical polymerization (oxidative coupling) starts with the oxidation of monomers to a cation radical and their coupling to form dications. The repetition of this process generates a polymer. All the classes of conjugated polymers may be synthesized by this technique [7].

Table 1 – List of some conductive polymers and corresponding common methods used for their syntheses.

Polymer	Method used	References
polycetylene	Chemical polymerization	[1]
polyaniline	Chemical polymerization or	[8, 9]
	Electrochemical polymerization	[10]
Polybutadiene	Inclusion polymerization	[11]
Polypyrrole	Chemical polymerization or	[12]
	Electrochemical polymerization	[13]
polystyrene	Concentrated emulsion polymerization	[14]

Moreover, conductive polymers show almost no conductivity in the neutral (uncharged/undoped) state but their conductivity can be increased several-fold by doping, which is the process of oxidizing (p-doping) or reducing (n-doping) a neutral polymer thus providing a counter anion or cation (i.e., dopant), respectively (Figure 1).

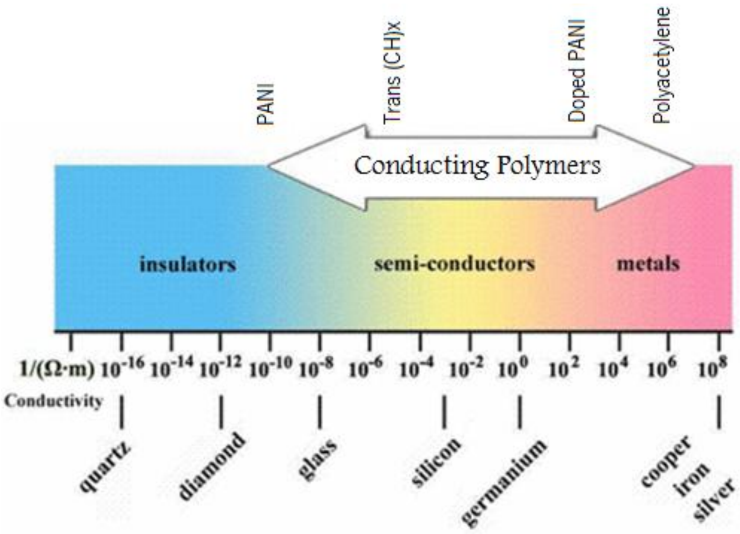

Figure 1 - Conductivity of electronic polymers. Conductivity increases with increased doping. [15]

Doping is accomplished by chemical methods of direct exposure of the conjugated polymer to a charge transfer agent (dopant) in the gas or solution phase; or by electrochemical oxidation or reduction and is dependent on oxidation potential. The doping is usually quantitative and the carrier concentration is directly proportional to the dopant concentration. Doping of conductive polymers involves random dispersion or aggregation of dopants in molar concentrations in the disordered structure of entangled chain and brils [4, 7]. Oxidation of the neutral polymer and following relaxation processes causes the generation of localized electronic states and a so-called polaron is formed. If now an additional electron is removed, it is energetically more favourable to remove the second electron from the polaron than from another part of the polymer chain. This leads to the formation of one bipolaron rather than two polarons. However, it is important to note that before bipolaron formation the entire chain would first become saturated with polarons [16]. Besides the increase in conductivity, doping in conductive polymers leads to some other interesting features which can be used in various technological applications (Figure 2).

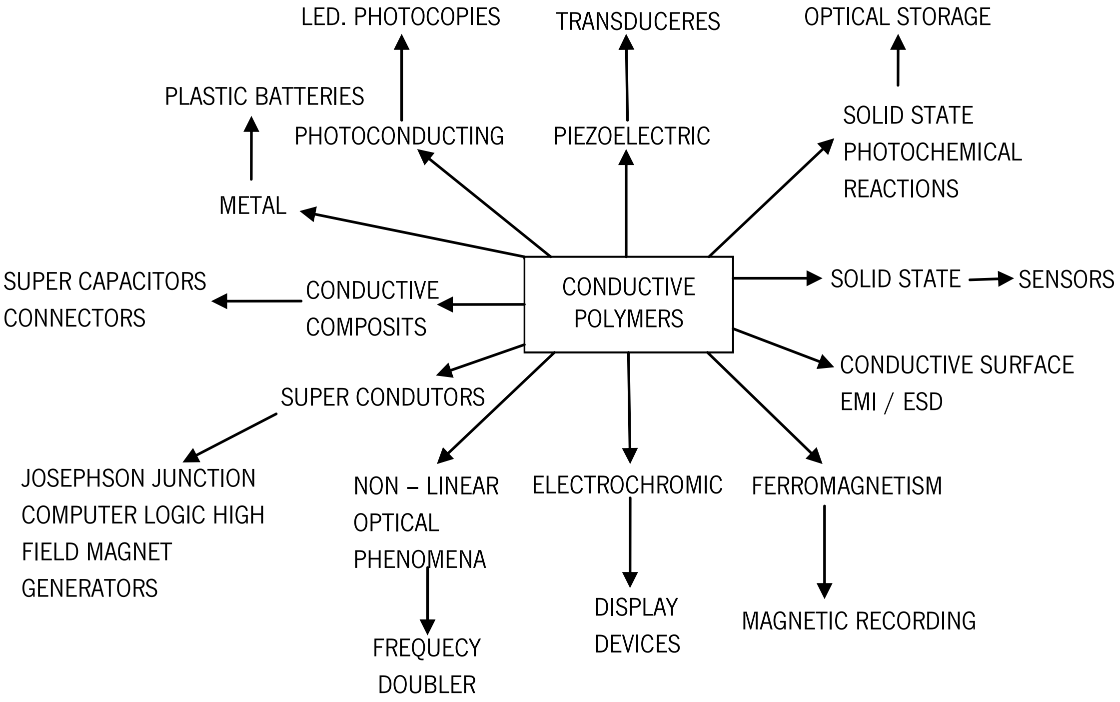

Figure 2 – Technologic applications of conductive polymers. Adapted from [7].

In the last few decades, given the unique combination of electrical, electrochemical and physical properties, the conducting polymers have received considerable attention due to their potential applications in electrodes, biosensors, batteries, antistatic coatings, gas sensors, membranes and light emitting diodes, transparent electrodes, electrical conductive, optical materials, biomedical applications, to name a few examples. [4, 17-19]

In photographic film development, Bayer and Agfa, initiated the development of the Baytron series of products based on poly(ethylenedioxythiophene) (PEDOT) doped with poly(styrene sulphonate) PSS [20]; polyacetylene, polythiophene, and an oligomer of thiophene were assayed for the development of thin-film organic field-effect transistors (OFETs) [20]. Another area that utilizes the optical and semi conductive properties of conductive polymers is solar-energy conversion, since they are strong absorbers of electromagnetic radiation over a wide frequency range [20]. Conductive polymers have been also considered for microelectronics applications, such as: Electronics Company Philips is involved in the development of “plastic chip” technology using conductive polymers; Mitsubishi Rayon in Japan has been producing a water-soluble sulfonated methoxyaniline polymer for use in e-beam lithography; and the use of polyaniline coatings for electroless deposition of copper connectors [2, 20].

In biomedical applications, the conducting polymers exhibit important advantages including biocompatibility, ability to entrap and controllably release biological molecules (i.e., reversible doping), ability to transfer charge from a biochemical reaction, and the potential to easily change the electrical, chemical, physical, and other properties of the conducting polymers to better suit the nature of the specific application. These unique characteristics are useful in many biomedical applications, such as biosensors, tissue-engineering scaffolds, neural probes, drug-delivery devices, and bio-actuators [4]. In recent times, studies involving the growth and control of biological cell cultures on conductive polymers for biotechnology applications have been described in the literature [21, 22]. It was envisaged that electrical and chemical stimuli can be used to address living cells in culture and thereby stimulate and regulate growth. Many studies have focused on nerve cells for neuroprostheses, bionic systems and neural repair devices, either in the peripheral nervous system or even for spinal chord regeneration [19, 23-25]. Conductive polymers have also been demonstrated to be very useful materials for tissue-engineering scaffolds and as actuators to operate as artificial muscles [26-28]. The electrical stimulation was found to promote favorable cell growth, including nerve cells, leading to the development of conductive polymers for a range of implant applications. [29-31]. Another application is in the area of biomedical monitoring. Here, a number of sensor fibers are built into garments and the sensor responses recorded, and in some scenarios automatically transmitted [2]

3.1. Polyaniline

Polyaniline (PANI) and the polymers derived from aniline are regarded as one of the most promising electrical conductive polymers due to their unique properties such as: their chemical versatility, stability, processability and low cost as well as its electronic, biological, and optical properties. [18, 32-35] A large number of studies have been carried out on the synthesis, structure, properties and applications of PANI [32].

PANI is one of the oldest conductive polymers known and was first synthesized by Letheby in 1862 by anodic oxidation of aniline in sulphuric acid and described as existing in different oxidative states (Figure 3), directly affected by the reaction conditions. It is a phenylene-based polymer with an amine group –NH on a polymer chain with a phenylene ring of both sides. The protonation and deprotonation occurs precisely due to the presence of the –NH group [7, 36].

The electrical conductivities of PANI range from 10^{-8} to 10^{2} S/cm. Depending on the conditions of preparation, the polymers may be soluble in various organic solvents or insoluble in any solvent. PANI can be produced as nanogranular powders, nanotubes, nanowires, micromats or microspheres and the dimensions of all the structures are determined by the molecular weight of the PANI chains, which is proportional to their length. These morphologies can be obtained as precipitates during the oxidation, as colloidal dispersions, or as films and layers on a variety of supports [33].

Besides the high conductivity, PANI has other interesting property, displays different colors when changing the conditions of pH or electrical potential.

3.1.1 Structure and Properties of Polyaniline

As mentioned above, PANI can occur in a variety of oxidation states that differ in chemical and physical properties.

The main oxidation states of PANI are represented in Figure 3. Leucoemeraldine (yellow) and pernigraniline (purple) are under the fully reduced state (all the nitrogen atoms are amine), whereas the fully oxidized state (all the nitrogen atoms are imine) include emeraldine (salt-green or base-blue) with the ratio –N-/N= of ~ 0.5 [36].

PANI can be doped by protonation without changing the number of electrons (oxidation/reduction) associated to the polymer chain. Thus the imine can be partially or fully protonated depending on the pH at which the polymer was exposed leading to the formation of doped PANI (conductive emeraldine salt). Upon further oxidation, a second redox process occurs, which yields a new insulating material, pernigraniline that also exists as a 'salt' and a base. The protonated pernigraniline is an important polymerization intermediate; its blue colour should not be mistaken for that of the emeraldine base, which is of a different shade (absorption maxima at 690 and 630 nm, respectively).

The deprotonation is reversible by treating the conductive emeraldine salt in neutral or alkaline media. This guides to a decrease of conductivity by ten orders of magnitude leading to emeraldine base. [33, 37, 38]

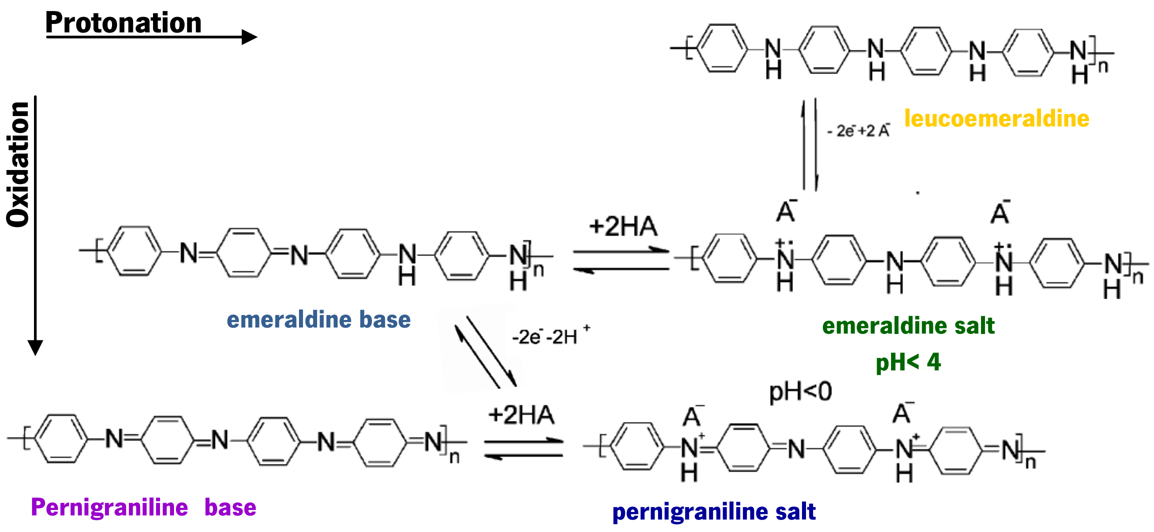

Figure 3 – Schematic representation of different oxidation states of PANI [16]. The colors are representative of the observable color of the polymer.

According to Sapurina *et al*, (2008) a PANI with a conductivity $>10^{-3}$ Scm^{-1} is produced only in strongly acidic media, pH <2.5. The oxidation of aniline in mildly acidic, neutral or even alkaline media yields non-conductive oligomers as products or the major components of the products. The pH drifts to lower values during the oxidation of aniline and the oxidation mechanism changes correspondingly during the oxidation. The reaction may pass through all three acidity phases in a single experiment; the oxidation of aniline in water in the absence of added acid is a typical example [33].

The most common methods used for preparation of PANI includes: dispersion polymerization of aniline in the presence of a matrix polymer in a disperse or continuous phase of a dispersion; Chemical in situ polymerization of aniline in a matrix or in a solution with a matrix polymer; Electrochemical polymerization of aniline in a matrix covering an anode; Polymer grafting to a PANI surface; Copolymerization of aniline with other monomers resulting in the formation of soluble aniline copolymers, which can be considered as a composite polymer [39].

Among these methods, chemical polymerization is favorable if considering the large-scale production of PANI [4]. It can be done using a variety of chemical oxidants, such as ($(NH_4)_2S_2O_8$), MnO_2, Cr_2O_4, H_2O_2, $K_2Cr_2O_7$, $KClO_3$, $FeCl_3$) in an acidic aqueous medium (HCl, H_2SO_4, $HClO_4$) [32, 40]. The main function of the oxidant agent is to remove a proton from a molecule of aniline, without forming a strong bond with both subtract intermediated or the final product. The oxidant agent should have a sufficient reduction potential for the oxidation of the monomer, but the amount to be used should be low enough to avoid the oxidative degradation of the produced polymer [33]. A common system for the production of PANI is the in situ chemical oxidation of aniline with ammonium peroxydisulfate (APS) in acid aqueous solution, this oxidation of aniline is exothermic and sulfuric acid and ammonium sulfate are by-products (Figure 4) [37].

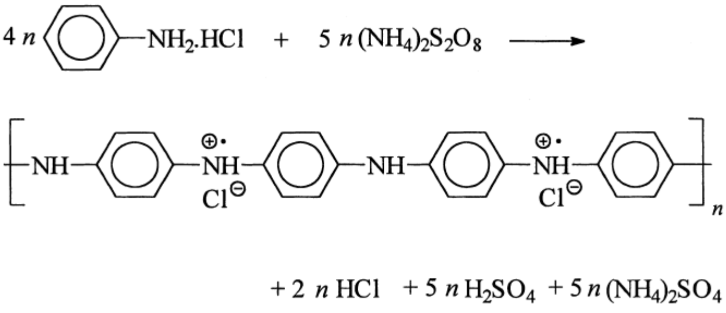

Figure 4 - Stoichiometry of aniline oxidation with APS to polyaniline (emeraldine) hydrochloride [37].

There are many important parameters, involved in PANI production, that can affect directly the PANI morphology and chemical and electrical properties including: the chemical nature of the oxidant agent, the nature of the acid protonating the aniline and the reaction intermediates during the oxidation, the pH, the concentrations of the reactants (especially of aniline and oxidant) and their molar proportions, temperature, solvent components (e.g. the organic component), the presence of additives (e.g. colloidal stabilizers, surfactants), templates added to the reaction mixture, etc [33].

3.1.2 Applications of Polyaniline

The combination of electrical properties, typical of semiconductors, with materials parameters characteristic of polymers, as been used for the development of “plastic” microelectronics, electrochromic devices, tailor-made composite systems, and “smart” fabrics [34, 37].

PANI has also been explored in the field of microelectronics. The combination of tunable chemical properties with the electronic properties of conductive polymer has a remarkable impact on the development of new sensors.

PANI membranes have been tested in pervaporation experiments, gas separation, metal recovery by electrodialysis, analytical ion-selective electrodes, pH sensors, enzyme immobilization, improvement of thermal stability, and trans-membrane redox reactions. Recent efforts in the design of PANI-modified or PPy-containing membranes have been aimed at applications in fuel cells [41].

Recently the demonstration of PANI’s biocompatibility [42], aside from conductivity, has sparked interest in its use for biomedical applications, including the development of artificial muscles [43-45], controlled drug release [46] or for the stimulation of nerve regeneration [19]. PANI was found to have good conductivity, low cytotoxicity and good biocompatibility allowing for the cell attachment and proliferation [47, 48].

4. Bacterial Cellulose

Cellulose, the most important skeletal component in plants, is the most abundant biopolymer on earth representing about 1.5 *10^{12} tons of the total annual biomass production [49]. It is composed of the linear homopolymer of β(1- 4)-linked D-glucose units covalently linked through acetal functions between the equatorial OH group of C4 and the C1 carbon atom (b-1,4-glucan) and can be obtained by different means (Figure 5): insolation from plants (most common); biosynthesis by different types of microorganisms; enzymatic in vitro synthesis; and the chemosynthesis from glucose derivatives [50, 51]. It is crystalline due to the high number of hydrogen bonds from the hydroxyl groups which hold cellulose chains together [52].

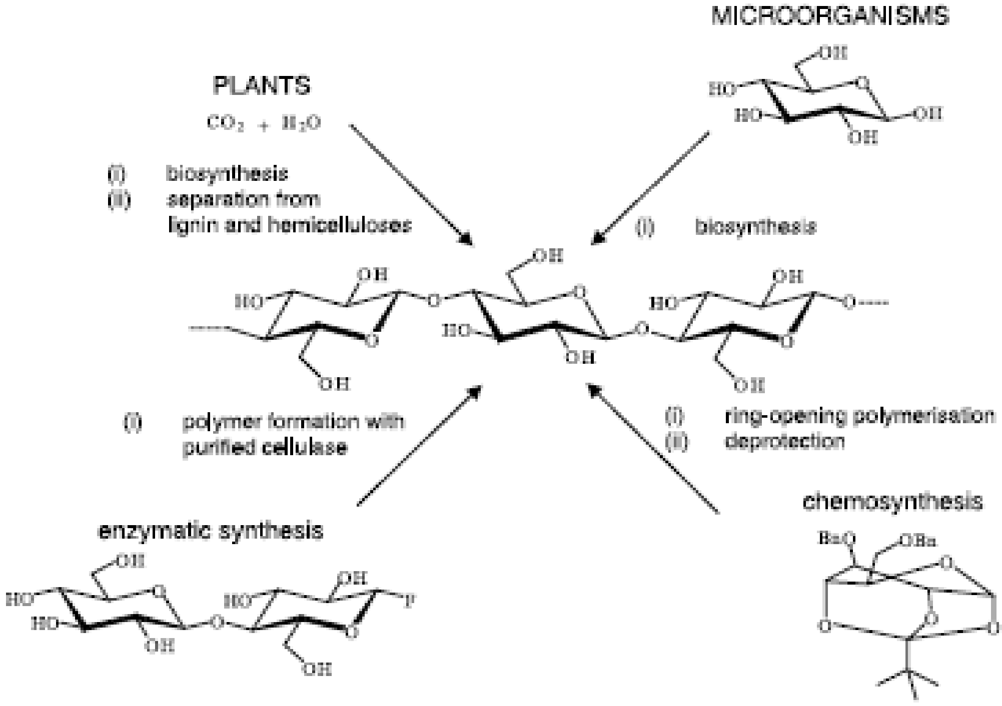

Figure 5 – Pathways to the cellulose [49].

The formation of cellulose by laboratory bacterial cultures is an interesting and attractive way to pure cellulose for both organic and polymer chemists. By selecting the substrates, cultivation conditions, additives, and finally the bacterial strain, it is possible to control the molar mass, its mass distribution, and the supra-molecular structure. Thus it is possible to control important cellulose properties, and also the course of biosynthesis (e.g. kinetics, yield, and other metabolic products) [50].

Both bacterial and vegetable celluloses have the same molecular formula being built up of β(1→4)–linked D-glucose units as referred above (Figure 6) but their physical and chemical features are quite different [53, 54]. Bacterial cellulose has developed into a field of study of its own (BC), as can be observed by the number of patents and publications worldwide (Figure 7) [53]. It is preferred over the plant cellulose as it can be obtained in higher purity free of lignin, pectin, hemicelluloses and other biogenic compounds (there is no need for chlorine chemical bleaching), and has higher polymer crystallinity [54, 55]. The degree of polymerization it is also different, about 13000-14000 for plants and 2000-6000 for bacterial cellulose [53]. It also has higher tensile strength and water holding capacity and the fibrils of bacterial cellulose are about 100 times thinner than that of plant cellulose, making it a highly porous material [54].

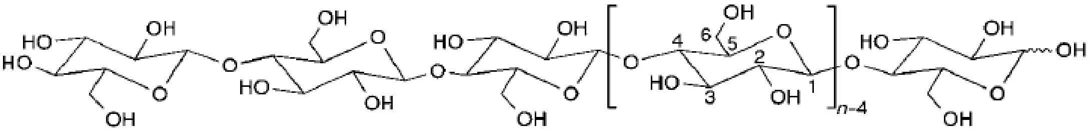

Figure 6 - Molecular structure of cellulose as a carbohydrate polymer generated from repeating B-D-glucopyranose molecules. (*n*=degree of polymerization)

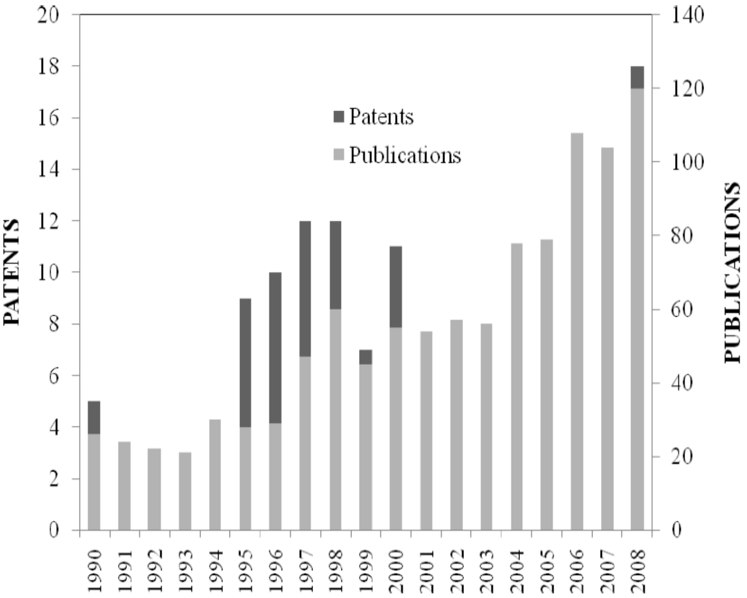

Figure 7 – Publications and patents on bacterial cellulose [53]

Bacterial cellulose can be produced by various species of bacteria (Table 2), such as those of the genera *Acetobacter, Agrobacterium, Psuedomonas, Rhizobium, and Sarcinathe.* The last one is the only genus of Gram-positive bacteria in the field [53]. Different strains producing microbial cellulose can be observed in Table 2 [54].

Table 2 - Different strains producing microbial cellulose [54]

Organisms (genus)	Cellulose produced	Biological role
Acetobacter	Extracellular pellicle Cellulose ribbons	To keep in aerobic environment
Achromobacter	Cellulose fibrils	Flocculation in wastewater
Aerobacter	Cellulose fibrils	Flocculation in wastewater
Agrobacterium	Short fibrils	Attach to plant tissues
Alcaligenes	Cellulose fibrils	Flocculation in wastewater
Pseudomonas	No distinct fibrils	Flocculation in wastewater
Rhizobium	Short fibrils	Attach to most plants
Sarcina	Amorphous cellulose	Unknown
Zoogloea	Not well defined	Flocculation in wastewater

Special attention has been given to the assembly of cellulose from *Gluconacetobacter xylinus* = *Acetobacter xylinum*, a Gram-negative, rod shaped bacteria. This is one of the best bacterial species for large-scale cellulose production [55] and it was first reported in 1886 by A.J. Brown.He observed that the resting cells of *Acetobacter* produced cellulose in the presence of oxygen and glucose [54].

The microfibrillar structure of BC was described by Mühlethaler in 1949. The author observed that the cellulose produced by *Acetobacter xylinum* occurs in the form of fibres. First, the bacteria secreted a structurally homogeneous slimy substance within which, after a short time, the cellulose fibers were formed. *Acetobacter xylinum* produces two forms of cellulose: (*i*) cellulose I, the ribbon-like polymer, and (*ii*) cellulose II, the thermodynamically more stable amorphous polymer [54].

Further, Hestrin and Schramm [56], intensively investigated the biochemistry of BC production by *G. xylinus*. The pH of the medium is 6 and the optimum growth temperature is 30°C. The static culture leads to the production of cellulose by the accumulation of a gelatinous membrane on the surface of the 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, rinkled appearance [53].

The differences between stationary and agitated culture are not only in terms of macroscopic morphology but also at various structural levels. While the fibril network remains the same, there are some differences in the structure of the 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 [57].

In terms of bacterial cellulose biosynthesis, the cellulose synthase is considered the most important enzyme. 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. 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 1.5nm 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 [53].

Existing relevant aspects that affect the bacterial cellulose production are: the carbon (mainly the glucose) and nitrogen sources and concentration, the air/liquid interface of the culture medium, the pH and temperature, the surface area of the fermentation system, the membrane properties (in static or agitated cell culture) and differences in the bacterial strains that also play an important role in the microstructure and production rate. Figure 8 shows a membrane produced by ATCC 10245 *G. Xylinus* strain [53]. The choice of the medium technique depends of the final destination of the biopolymer.

Figure 8 - BC pellicle produced by ATCC10245 *G. xylinus* strain in static culture [53]

In terms of properties BC is characterized by a tridimensioal (3D) ultrafine network structure [50]. When formed in static culture, the randomly assembled ribbon-shaped fibrils are less than 100 nm wide and composed of elementary nanofibrils, aggregated in bundles with lateral size of 7-8nm. The crystallinity degree of BC is in the range of 60-90% [53]. According to Iguchi [58], a BC pellicle obtained after 7 days of culture and air-dried at 20°C and low pressure, presents a Young's modulus of 16,9 GPa, tensile strength of 256 MPa and elongation of 1,7% . However, when the pellicle is dried through the heat-press method with an excess of pressure (490 – 1960 kPa), the tensile strength and elongation tend to decrease, while the Young modulus remains constant.

BC is highly porous (around 94%) with micro-channels of different size through which solute diffusion occurs and highly hydrophilic, holding over 100 times its weight in water. The method of drying has been shown to affect the BC porosity. The freeze-drying technique has been reported as the most effective method to preserve the porous structure [49, 53]

4.1 Applications of Bacterial Cellulose

Due to its characteristic, an almost inexhaustible polymeric raw material, friendly to the environment, biocompatible, biodegradable, high water-holding capacity and with remarkable mechanical strength (Young's modulus of 15-30 GPa), this polysaccharide is a very useful natural material which has a lot of potential and it has been used in many applications in the most random areas [49, 50, 59, 60].

Once BC is biocompatible and highly pure it has been studied extensively for biomedical applications, especially for use as scaffolds in tissue engineering including drug delivery, vascular grafts, cartilage, neural regeneration and wound dressing [53].

The Biofill (BioFill Produtos Biotecnológicos, Curitiba, PR, Brazil) membranes were the first bacterial cellulose membranes to be used in clinical trials, creating a new wound healing system. They also developed products such as Bioprocess used to treat cases of second and third degree burns, ulcers and as temporary substitute for human skin; and Gengiflex used for recovery of periondontal tissues, dental implants and guided bone regeneration, alone or in association with osteointegrated implants, proving a good alternative for guided tissue regeneration [53, 57]

Svensson and colleagues [61] utilized native and chemically modified BC as a substrate for primary bovine and human chondrocytes culture, aiming at constructing a cartilage tissue with native mechanical properties. The authors found that BC scaffolds support the growth of chondrocytes, allowing cell migration and ingrowth.

Klemm *et al.*, (2001) [49] investigated the application of patented BC tubes (BASYC - BActerial SYnthesized Cellulose) as microvessel endoprosthesis for end-to-end anastomosis procedure, using the carotid artery of a white rat and demonstrated that there was no rejection reaction.

Putra *et al.,* (2008) [62] described a simple technique that allows obtaining a tubular – BC gel with desired length, inner diameter and thickness, along with an oriented fibril structure. This technique requires a shorter cultivation time, as compared to the methodology described by Klemm *et al.*

In the last few years BC has also been vastly explored for the food and electronics industries and for papermaking. In the food industry, BC is used in the production of coconut cream, low fat ice cream, snacks, sweets, thickener, stabilizer, texture modifier, and serum cholesterol-lowering [63]. Nata de Coco, obtained by the static fermentation of coconut wastewaters, became one of the first commercially available products of BC, gaining notable popularity [53].

In electronics, due to the demand of low-cost manufacturing techniques, BC has been tested for display devices, coatings, and audio components. Organic light emitting diodes (OLEDs) are an emerging technology based on the design of light-weight, flexible thin film devices that use electroluminescent organic materials (flexible displays). Several efforts have also been focused on achieving electronic display screens that combine the desired properties of paper, with the dynamic capability of digital screens [53].

Along with the recognized advantages of their use in several consumer products, BC based flexible displays can be used to develop devices for therapeutic purposes such as photodynamic therapy, to treat skin cancer and other diseases.

According to Yano *et al,* (2005) [64], BC exhibits a high optical transparency making it a suitable material for display devices, coatings and lenses.

Also, the first audio speaker diaphragms using microbial cellulose were developed by the Sony Corporation. The excellent dimensional stability of microbial cellulose gives rise to a sound transducing membrane which maintains high sonic velocity over a wide frequency range. This makes it the best material to meet the high standards for optimum sound transduction [53].

In papermaking Mormino and Bungay [65], in 2003, used a novel fermentation system to produce composites of BC and paper. Gostomski and Bungay [66], in 2002, described the production of BC using a horizontal bioreactor containing half-submerged, flat, circular disks mounted on a central shaft. Overall, the composites showed strengths more than ten times that of controls composed only of BC. The studied system was proposed to allow expanding the market for the recycled material and might be even more valuable as a low-cost strengthener or bulking agent for other applications of the cellulosic gel or dried sheets that may need the extra strength [66]. Companies such as Mitsubishi Paper Mills in Japan are also investing a lot of resources in developing microbial cellulose for paper products. [53]

Thanks to the emerging of nanotechnology, recent studies of bio-based BC nanomaterials have been reported. Tailoring how cellulosic interfaces are constructed at the nanoscale may provide the opportunity to develop new and better materials and products. Also, the cellulose‘s chemical characteristics provide it with a rich variety of options for chemistry and engineering for material applications. Its relative chemical purity, thus obviating the purifications steps and the nanometer range of the fiber, facilitate its exploitation. [53]

Cristian *et al,* (2009) [67] developed a bio-inspired bottom-up process to produce bacterial cellulose–starch self-assembled nanocomposites. Potato and corn starch were added into the culture medium and partially gelatinized in order to allow the cellulose nanofibrils to grow in the presence of a starch phase. The BC-starch gels were hot pressed into sheets that had a BC volume fraction higher than 90%. During this step starch was forced to further penetrate the BC network. This process takes advantage of the way some bacteria extrude cellulose nanofibres and of the transport process that occurs during the gelatinization of starch. The self-assembled BC–starch nanocomposites showed a coherent morphology; the crystallinity of BC was preserved in spite of the presence of starch, hence the mechanical properties of the nanocomposites showed no significant decrease. This bottom-up technique seemed to be an adequate approach to manufacture BC nanocomposites allowing the preservation of the typical network of cellulose fibres as there was no need to disintegrate the BC gel in order to combine it with a second phase and [53, 67, 68]

Won-Il Park *et al,* (2007) [69] incorporated cellulose whiskers into nanofibers of polyethylene oxide (PEO) by using electrospinning process. Electrospinning is a fast and simple process driven by the electrical forces on the surface of polymeric fluids, producing polymer filaments using an electrostatic force. This electrospinning technique can serve various purposes, such as the fine control of the fiber diameters, the production of a defect-free or defect-controllable fiber surface, and the formation of continuous single nanofibers. Thus process yield to BC/PEO nanocomposite fibers with a diameter of less than 1 μm. The rod-like BC whiskers, prepared by the acid hydrolysis, were 420 nm long and 11 nm wide, with a height of 10 nm. The whiskers were well embedded and aligned inside the fibers, even though they were partially aggregated in some of the fibers. They suggest that the incorporation of the cellulose whiskers was efficient in enhancing the mechanical properties of the electrospun fibers [53, 69].

The CB has also been proposed to be used for membrane fuel cell (hydrogen), electronic paper (e-paper) ultrafiltration, pervaporation and dialysis membranes and membranes for recovery of oil and mine [70].

CHAPTER II.

STATE OF THE ART

1. Cellulose-based Conductive Materials

Due its unique structure and properties in terms of its purity, ultrafine network, high mechanical stability and low density, many studies have been performed in order to achieve the ideal cellulose-based conductive materials. Most of them involve the ''in situ'' chemical polymerization of aniline or pyrrole in the presence of cellulose or bacterial cellulose. In Table 3 is summarized some of that studies.

Table 3 – Review of some studies on cellulose-based conductive materials.

Matrix		Oxidant agent	Doping	Medium	Temperature (ºC)	pH	Conductivity (S/cm)	Ref.
Cellulose fibers	PANI	APS and FeCl3	–	HCl	Room temp.	–	$5.6*10^{-4}$ to $6.0*10^{-4}$	[71]
	Polypyrrole						$2.1*10^{-3}$ to $2.6*10^{-1}$	
Chitosan	PANI	APS	–	HCl	25	0	$9.63*10^{-2}$	[34]
Cellulose fibers	PANI	APS	–	HCl	20	<3	0.41	[72]
BC	PANI	APS	–	HCL	Room temp.	<3	$5*10^{-2}$	[9]
Cellulose blends	PANI	–	DIOHP	Destilled water	160	–	$1*10^{-3}$	[73]
BC	PANI	APS	DBSA	Ethanol/water	25	5.5	$1.61*10^{-4}$	[74]
BC	PANI	APS	–	HCl	Room temp.	<3	$3.0*10^{-2}$	[17]
BC	PANI	APS	p-TSA	HCl	Room temp.	3	1.3	[8]

Laska *et al,* (1997) [73] evaluated the conductivity of different PANI blends doped with phosphoric acid diesters in convention polymers (PVC, polystyrene, poly(metylmethacrylate)) and in cellulose derivates. According to these authors the cellulose/Pani blends shows a conductivity of $1.0*10^{-3}$S/cm. The blends of PANI seem to be particularly attractive by combining relatively high conductivity with good mechanical properties.

Li *et al,* (2002) [75] developed a method to prepare surface-conductive glass fibers. The method consisted on: (a) calcination of the fiber to remove the attached organic material; (b) activation of the fiber to generate a maximum density of silanol groups by immersion in a HCl aqueous solution; (c) formation of a stable silane monolayer through the reaction of 3-bromopropyltrichlorosilane with the hydroxyl groups on the surface of the glass fiber; (d) the functionalization of the "self-assembly" monolayer through aniline substitution; and (e) the surface oxidative graft polymerization of aniline via the covalently immobilized aniline sites. The method produced a smooth, homogenous thin layer of PANI with strong adhesion on the surface of the glass fiber. The surface conductivity of the composite had a value of about 6.7 S/cm.

Johnston *et al*, (2005) [12] prepared various composite conductive polymers by direct polymerization of polypyrrole and PANI on paper sheets using ferric chloride as the oxidant agent. They aimed to combine the paper sheets/products properties with the chemical and electrically conductive properties of the conductive polymers. Conductivities of up to 6 S/cm for paper-polypyrrole composites and up to 2*10-3 S/cm for paper-PANI composites were obtained.

There are a few publications focused on the combination of BC with carbon nanotubes. Yoon *et al*, (2006) [76] described a method to produce electrically conductive polymeric membranes, prepared by incorporating multiwalled carbon nanotubes (MWCNTs) in bacterial cellulose by dipping cellulose pellicles in a aqueous MWCNT dispersion containing a surfactant. The experiment showed that MWCNTs were strongly adhered to the surface of the bacterial. The electrical conductivity of pure MWCNTs was about $2.3*10^{1}$ S/cm and the final conductivity of the cellulose/MWCNT composite was found to be approximately $1.4*10^{-1}$ S/cm. The incorporation process reveals to be a useful method for dispersing MWCNTs in an ultrafine fibrous network structure and also for enhancing the electrical conductivity of the BC membranes [76].

Van den Berg *et al*, (2007) [77] built a system consisting of a mixture of cellulose whiskers suspension with polyaniline (PANI) solution and a poly(p-phenylene ethynylene) (PPE) derivative with quaternary ammonium side chains. Cellulose whiskers with a typical average diameter of 20 nm, an avarage length of 1–2 mm, were combined with the positively charged p-conjugated polymers to form stable dispersions in polar solvents such as formic acid. Thin films were produced by solution casting. These researchers obtained nanocomposites that combined the electronic characteristics of the conjugated polymers with the outstanding mechanical characteristics of the cellulose scaffold [77].

Kelly *et al*, (2007) [71] produced polypyrrole and polyaniline conductive polymer composites in which individual Kraft paper pulp fibres have been fully encapsulated with polypyrrole and polyaniline by direct polymerisation of the respective monomers using ferric chloride and ammonium persulfate as the oxidants. The electrical conductivity of the prepared hybrid materials ranged from $5.6*10^{-4}$ S/cm to $6.0*10^{-4}$ S/cm for PANI composites and $2.1*10^{-3}$ S/cm to $2.6*10^{-1}$ S/cm for polypyrrole composites.

Tiwari and colleagues (2007) [34] used chitosan to fabricate an anisotropic graft conductive copolymer synthesized by grafting of polyaniline on to chitosan using APS as an initiator in acidic medium. The electrical conductivity of the composite was shown to increase with the extent of grafting onto the chitosan backbone and with the decrease of pH, reaching a conductivity of $9.63*10^{-2}$ S/cm at a pH=0. According to these authors this biopolymer could be exploited for chemical and biosensor applications due to its good processability, improved solubility, high mechanical strength and controlled electrical properties. With conductivities as high as $4.03*10^{-3}$ S/cm, the optimum grafting efficiency was found at $[(NH_4)_2S_2O_8]$ $12.5*10^{-2}$ g/l, [aniline] $1.5*10^{-2}$ g/l, [hydro-chloric acid] 0.5 g/l, [chitosan] 1.0 g/l, temperature 25±0.2°C [34].

Stejskal and co-workers (2008) [72] coated cellulose fibers with protonated polyaniline (PANI) during the oxidation of aniline hydrochloride with APS in an aqueous medium. The conductivity increased from $4.0*10^{-14}$ S/cm to 0.41 S/cm after coating the fibers with PANI. The subsequent reaction with silver nitrate results in the decoration of PANI-coated cellulose fibers with silver nanoparticles of about 50 nm average size. The emeraldine coating changed to the pernigraniline state during the latter process and, consequently, the conductivity of the composite decreased from 0.41 S/cm to 4.1×10^{-4} S/cm, despite the presence of silver. These composites are also intended for applications outside the field of conducting materials.

Zun-li Mo *et al,* (2009) [17] investigated a series of cellulose–PANI conductive composites prepared by chemical oxidative polymerization of aniline with native cellulose activated by various di-basic and monobasic acids. In that study, the composites prepared using the di-basic acids exhibited more favorable conductivity than the composites prepared using the monobasic acids. Moreover, the content of PANI and consequently the conductivity increased with increasing of activation time, however, for activation time higher than 50 min, the conductivity decreased because of the formation of aggregated PANI particles. Both the PANI content and the electrical conductivity increased with an increase of the amount of aniline, and reached the maximum values $3.0*10^{-2}$ S/cm at the 0.5 g aniline, respectively. The acids were able to successfully activate cellulose and lead to the improvement of the accessibility and reactivity of the O–H groups. Results from thermal analysis assays indicated that the composites were highly stable compared to pure cellulose [17].

Qian *et al,* (2010) [78] prepared PANI-coated conductive paper. Different pulps were used to evaluate the influence of pulp type on the conductivity of PANI-coated paper. The amounts of PANI coat on chemical pulps were higher than those coated on high yield pulps. The conductivity presented a significant positive linear correlation with the amount of coated PANI. Also, the beating degree of the pulp seemed to have an insignificant effect on the conductivity of PANI coated conductive paper.

Nystrom *et al,* 2010 [79] analyzed the coating of individual fibers of wood-based nanocellulose with polypyrrole using *in situ* chemical polymerization. The results revealed that it is possible to manufacture an electronically conductive high-surface area composite material composed of microfibrillated cellulose and polypyrrole by direct chemical polymerization of pyrrole onto wood-derived nanofibers in hydrogels without the need for sophisticated and time-consuming drying techniques such as solvent-exchange drying or lyophilization. The dry composite has a specific surface area of 89 m^2/g; a conductivity of 1.5 S/cm; is electrochemically active; and exhibits an ion-exchange capacity for chloride ions of 289 C/g. These results give rise to new possibilities regarding the large-scale production of inexpensive paper-based materials for energy storage as well as electrochemically controlled extraction and separation of biologically interesting compounds [79].

2. Overview on Electrical Conductive Bacterial Cellulose-graft-Polyaniline

Despite the previously described advantages of the use of PANI for the development of conductive materials, this polymer has known limitations in solubility and mechanical properties. To compensate these weak properties, the PANI is normal incorporated in other materials. Thus, in general, grafting of PANI into a flexible matrix, such as bacterial cellulose, could result in a material with good processability, good mechanical properties, biocompatility and good/stable electrical conductivity.

A literature review shows that only this year (2011), four independent published reports focusing on the preparation of electrically conducting blends of bacterial cellulose as the polymer matrix, and intrinsically conducting polymer as the conductive filler were found. Marins *et al* (2011) [74] prepared conductive composite membranes of bacterial cellulose (BC) and polyaniline doped with dodecylbenzene sulfonic acid (PANI.DBSA) by means of *in situ* chemical polymerization of aniline in the presence of hydrated BC. The polymerization was performed with ammonium peroxydisulfate as the oxidant agent and different amounts of DBSA. The experiment showed that the highest electrical conductivity value 1.88*10^{-4} S/cm was achieved by using a DBSA/aniline molar ratio of 1.5 (because this condition provided a better penetration of PAni.DBSA chains inside the hydrated BC sheet). Higher amount of DBSA resulted in a DBSA layer at the BC surface, which avoid the penetration of the polyaniline chains inside inside the hydrated BC sheet. This behavior resulted in a decrease of the bulk electrical conductivity. The *in situ* polymerization gives rise to conductive membranes with the surface constituted by different degree roughness confirmed by SEM micrographs and Small angle X-ray scattering (SAXS) measurements.

Weili Hu *et al* (2011) [9] synthesized polyaniline/bacterial cellulose (PANI/BC) conductive nanocomposite membranes by *in situ* oxidative polymerization of aniline using ammonium persulfate as an oxidant and BC as a template (Figure 9). The authors found that PANI nanoparticles deposited on the surface of BC to form a continuous nanosheath by taking along the BC template, which greatly increases the thermal stability of BC. The content of PANI and the electrical conductivity of composites increased with increasing reaction time from 30 to 90 min. However, prolonged reaction times lead to a decreasing in conductivity because of the aggregation of PANI particle. The results also indicated that BC is successfully activated by acids, and the intermolecular hydrogen bands are broken, which helps to form the uniform dispersion of PANI in the nanofibrous membrane. The electrical conductivity of the membranes can achieve the value of 5.0*10^{-2} S/cm and the Young's modulus the value of 5.6 GPa and tensile strength of 95.7 MPa. Moreover, the electrical conductivity of the membrane is sensitive to the strain.

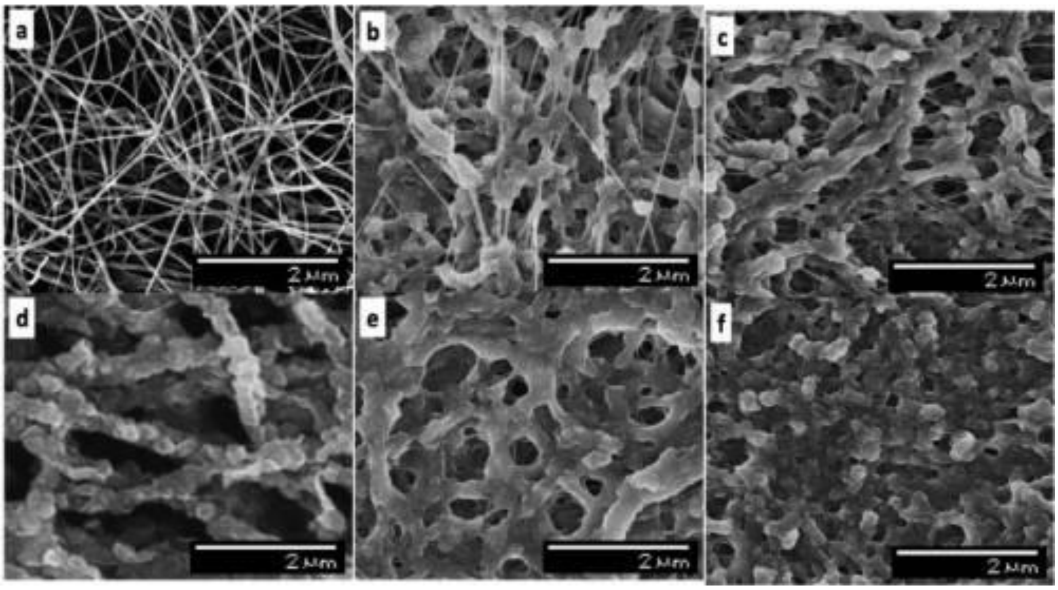

Figure 9 – FE-SEM images of a) pure BC and the PANI/BC composites produced with different polymerization time: b) 30 min, c) 60 min, d) 90 min, e) 120min and f) 180 min [9].

Byoung-Ho Lee *et al* (2011) [8] produced polyaniline conductive polymeric nanocomposite films with bacterial cellulose fibers by means of chemical oxidative polymerization of aniline. APS was used as oxidant and p-TSA as dopant. The experiment showed that in nanocomposite film, the bacterial cellulose was fully encapsulated with polyaniline spherical spheres by direct polymerization of the respective monomers using the oxidant and dopant. These nanocomposite films exhibited the inherent properties of both components. The oxidant and dopant had a significant effect on the electrical conductivity and thermal stability of the nanocomposite films. The results revealed the fine globular structure of the polyaniline on the nanocomposite films with average grain sizes ranging from 100 to 200 nm. X-ray photoelectron spectroscopy (XPS) revealed a higher doping level of the nanocomposite films doped with p-TSA dopant. The PAni obtained was thermodynamically stable. The calculated electrical conductivities were 1.3 S/cm for the BC/PANI using p-TSA dopant and 0.8 S/cm for BC/PANI not doped. Therefore, the doping level affects the electrical conductivity

CHAPTER III.

SYNTHESIS AND CHARACTERIZATION OF ELECTRICAL CONDUCTIVE BACTERIAL CELLULOSE-GRAFT-POLYANILINE

1. Material and Methods

The most important features of polyaniline are its good environmental, thermal and chemical stability, high electronic conductivity, redox and ion-exchange properties, and ease of preparation from common chemicals [33]. These characteristics, in conjunction with bacterial cellulose fibre properties (highly homogeneous structure, high moldability *in situ*, high water-holding capacity, biocompatibility and remarkable strength) allows the formation of a BC conductive composite that can guide to many applications. For neuronal tissue engineering, a suitable biomaterial should provide or support initial mechanical stability, cell distribution and good tissue biocompatibility. A electro-conductive bacterial cellulose composite which is expected to show conductivity and biocompatibility together with sufficient mechanical strength, has been the subject of study.

1.1. Materials

Gluconacetobacter xylinus (ATCC 53582), glucose, peptone, yeast extract, hydrated sodium phosphate dibasic ($Na_2HPO_4.H_2O$), Citric acid mono hydratated, sodium hydroxide (NaOH) and hydrogen chloride (HCl) were used to prepare the bacterial cellulose. Epycloridrine was purchased from Fluka and was used in this work as initiator in the CB modification process. Aniline and aniline hydrochloride, used to modify the surface of BC, were purchased from Sigma-Aldrich. Absolute ethanol and N-methylpyrrolidinone (NMP) was used to remove the free aniline on the BC. Ammonium peroxodisulfate of Sigma-Aldrich and Iron (III) chloride of Panreac were used as catalyst on the polymerization step. Sodium (meta)periodate, purchased from Sigma-Aldrich, and Sodium bisulfate from Acros, were used for the sulfonation of the BC.

All reagents and chemicals were of analytical grade and were used as received, excluding aniline which was distilled before use. All aqueous solutions were prepared using distilled water.

1.2. Methods

1.2.1. Synthesis and Preparations of Bacterial Cellulose

All BC was produced using the strain ATCC 53582 of *Gluconacetobacter xylinus* in static culture at 30°C for a period of two weeks. The culture media used was Hestrin Schramm [56], which consisted in the dissolution 20 g glucose, 5 g peptone, 5 g yeast extract, 3.38 g Na2HPO4.H2O and 1.5 g citric acid mono hydrated, in 1000 ml of distilled water. The pH of the solution was adjusted to pH 5 using HCl. *G. Xylinus* grown in these Hestrin–Schramm medium with a pH 5.0.

The BC discs were obtained using a 6-well Cell Culture Plate. When the BC discs reached the maximum thickness they were removed from the plates, rinsed with distilled water and immersed in NaOH 4% solution overnight. This step assured the destruction of the G. xylinus cells and the removal of the remaining culture media still present in the BC. Afterwards the processed BC was washed until it reached the pH of distilled water (5.5). After washing, part of BC was freeze-dryed and kept in desiccators at room temperature and the other part was stored in distilled water until further use [80].

1.2.2. Preparation of Polyaniline

Polyaniline in the esmeraldine salt form was synthesized according to the method described by Stejskal *et al* [37]. Distilled aniline 0.2 M was oxidized with 0.25 M ammonium peroxydisulfate (APS) in aqueous medium. For that two solutions were prepared: 50 ml solution of aniline dissolved in acidic aqueous media (0.1 M HCl); and 50ml of solution of APS also dissolved in acidic aqueous media (0.1 M HCl). Both solutions were kept at room temperature for 1hour, then mixed in a beaker, briefly stirred, and left at rest to polymerize. In the next day, the PANI precipitate was collected on a filter washed with three 100 ml portions of 0.2 M HCl and similarly with acetone. Polyaniline powder was dried in air and then in oven at 60°C. Then, the PANI was kept in desiccators at room temperature.

1.2.3. Bacterial Cellulose Modification

In this stage, three different methods were studied (Figure 10): direct polymerization of CB using aniline; polymerization by initial modification of the BC surface; and the sulfonation of BC.

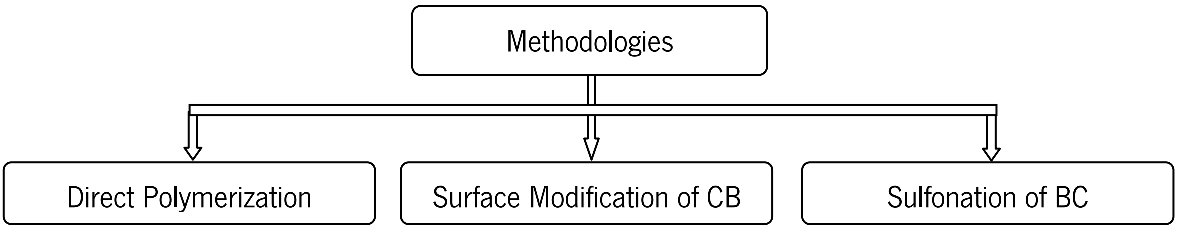

Figure 10 - Flowchart of the different approaches used for the production of BC-graft-PANI.

1.2.3.1. Direct Polymerization

In the direct polymerization, CB-graft-PANI was synthesized by oxidative polymerization of aniline in the presence of hydrated BC (Figure 11) [17]. For that, distilled aniline was dissolved in 50 ml aqueous HCl solution containing 100 mg of BC. After the BC was stirred for 1 hour, 50 ml of oxidant solution APS (0.125 M) was added dropwise under magnetic stirring. The reaction mixture was kept under continuous stirring for different polymerization times. In the preparation of the solutions, HCl 0.1 M was used. The polymerization of aniline was carried out at room temperature and using different ratios (BC:ANI) and different times in order to acquire the optimum conditions for grafting PANI onto the BC [34]. At the end of each reaction, the obtained products were washed with distilled water to remove the byproducts and remaining reagents. The samples were stored in desiccators after freeze-drying.

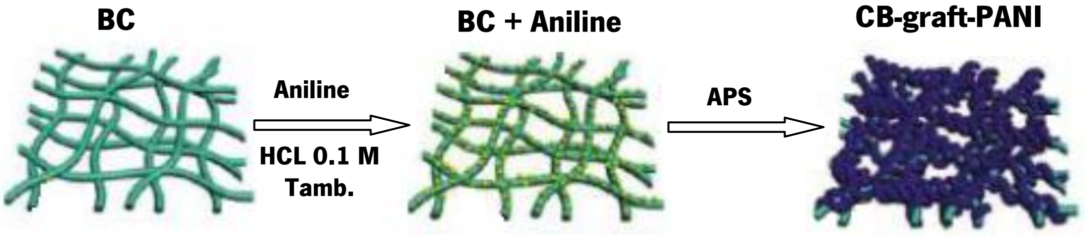

Figure 11 – Schematic representation of the process of the formation of CB-graft-PANI composite [9].

Table 4 - Designation and composition of the different BC produced by *in situ* chemical polymerization.

Designation	CB:ANI	Polymerization Time (h)
1:1 (6h)	1M:1M	6
1:5 (3h)	1M:5M	3
1:5 (6h)		6
1:5 (9h)		9
1:5 (12h)		12
1:5 (24h)		24
1:10 (3h)	1M:10M	3
1:10 (6h)		6
1:10 (9h)		9
1:10 (12h)		12
1:10 (24h)		24

1.2.3.2. Surface Modification of Bacterial Cellulose

This method was used to covalently attach the PANI to the surface of the BC nanofibers.

First, the surface of the BC nanofibers was decorated with epoxy functional groups via reaction with epichlorohydrin (Figure 12). For that, 100 mg of BC was hydrated with 132 ml of NaOH overnight. The solution was heated to 60°C and 960 μl of epichlorohydrin was added to the solution and stirred gently during 2 hours. Then, the BC was washed until it reached the pH of distilled water. After washing, the pH of BC was increased to 12 using NaOH (50% (w/v)) in 132 ml of distilled water. The blend was heated again to 60°C and 5ml of ammonia hydroxide (25%) was added and then stirred during 2 hours. After the modification step the BC was washed repeatedly to the pH of distilled water *(modification step)* [81].

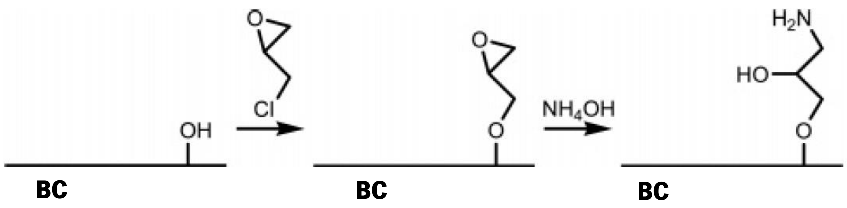

Figure 12 – Bonding of BC with epichlorohydrin. Adapted from [81].

The modified BC was placed in a solution of aniline hydrochloride (with 80 mg of aniline 50 ml distilled water) at room temperature and stirred during 2 hours *(activation step)*. The resultant BC was washed with 50 ml of N-metilpirrolidone (NMP) overnight. Then BC was washed with ethanol for 2 hours and after repeatedly with distilled water.

Ultimately, the *polymerization step*, BC was submerged in 50 ml of aniline hydrochloride solution (80 mg in 50 ml of distilled water), under constant stirring and slowly adding the 50ml of ammonium persulfate (176 mg in 50 ml of distilled water). The solution was left to react while stirring at room temperature during different periods of time and in the end, the BC was washed several times with distilled water [34]. Different activation and polymerization times were used.

Table 5 - Composition of the different BC produced by surface modification method.

Designation	**Activation time (h)**	**Polymerization time (h)**
A2hP2h	2	2
A2hP6h		6
A2hP12h		12
A2hP24h		24
A4hP2h	4	2
A4hP6h		6
A4hP12h		12
A4hP24h		24

To allow a better visualization, a flowchart representative of the methods is presented in Figure 13.

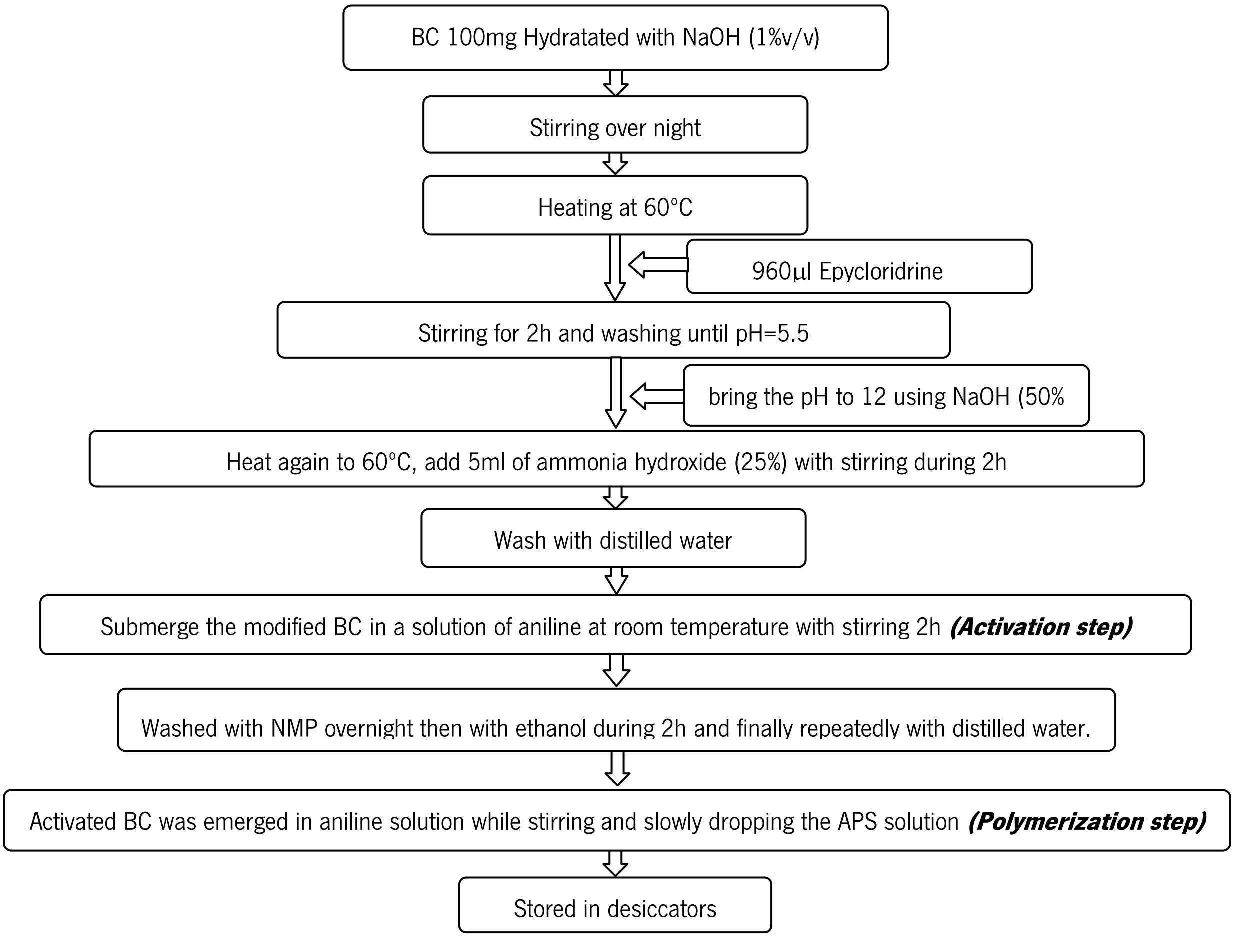

Figure 13 - Flowchart of the production of electrical conducting bacterial cellulose-graft-polyaniline by means of surface modification method

1.2.3.3. Sulfonation of Bacterial Celullose

The first step consist of the sodium periodate oxidation of CB. The CB discs were treated with $NaIO_4$, both with the same molecular ratio, in 75 mL of experimental volume in a 100 mL flask. The solution was stirred at 50°C in the dark, during different times to attain different degrees of oxidation. The resultant product is then washed with distilled water to remove the spent oxidant.

Samples were heated 1 hour before the assay (before the addition of ($NaIO_4$). In the second step, corresponding to the sulfonation of 2,3-dialdehyde CB, the oxidized CB react with sodium bisulfate ($NaHSO_3$) and stirred during 6 h and 24 h at room temperature (Ratio CB : $NaHSO_3$ $\rightarrow$ 1 : 2.3) [82]. The first and second steps were done in the same day avoiding the degradation of CB.

The modification of BC by periodate oxidation followed by sulfonation is represented in Figure 14.

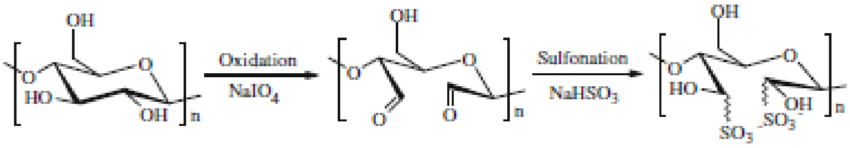

Figure 14 – Oxidation and sulfonation of CB [83].

The third step was the polymerization of sulfonated CB using $FeCl_3$ (Figure 15). To a volume of 100 ml at a pH of 4, using HCl and sulfonated CB: a) iron (III) chloride (Ratio-1 mol BC:10 mol aniline:20 mol $FeCl_3$) was added and the solution was stirred during 1 hour and after that aniline was added and stirring during 6h. The final BC was washed with distilled water and at the end with HCl 0.1M; or b) iron (III) chloride (ratio - 1aniline:2mol$FeCl_3$) was added to the solution with BC, stirring for 1 hour. Then BC was washed with HCl 0.1 M. Finally aniline was added and stirred for 6 h.

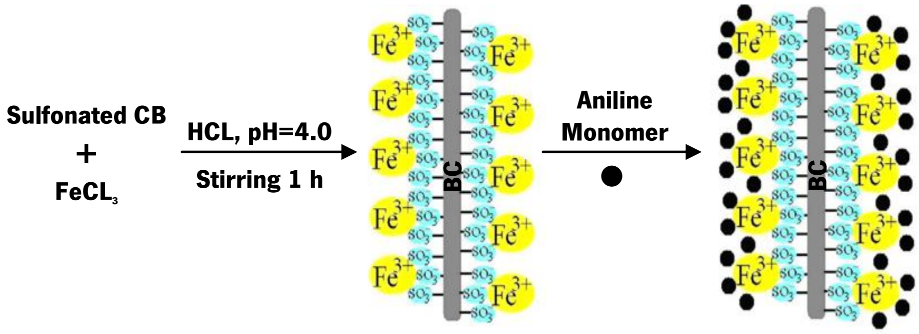

Figure 15 - Schematic illustration of the possible polymerization mechanism on modified CB using $FeCl_3$ [84].

2. Characterization of Electrical Conductive Bacterial Cellulose-graft-Polyaniline

With the aim of achieving the ideal method for synthesizing this composite and attain the aniline necessary for an efficient polymerization reaction the study of electrical behavior of the different samples was performed.

To complement this study, Fourier-transformed infrared (FTIR) spectroscopy was used in order to confirm the presence of specific chemical changes of functional groups that occurred in the structure, indicating that polymerization reaction occurred on the BC matrix.

The obtained electrical conducting bacterial cellulose-graft-polyaniline with optimized conditions was then characterized in terms of morphology by scanning electron microscopy (SEM), differential scanning calorimetry (DSC), thermalgravimetric analysis (TGA) and MTS assay.

2.1. Conductivity

Conductivity is a measure of electrical conduction and thus a measure of the ability of a material to pass a current. [4]

Electrical conductivity (σ) is defined as the inverse of the resistivity (ρ). This means a high resistivity is the same as a low conductivity, and a low resistivity is the same as a high conductivity:

$$\sigma \equiv \frac{1}{\rho}$$ [85]

Surface resistance, *Rs*, is defined in literature as the ratio of a DC voltage *U* to the current, *I* flowing between two electrodes of specified configuration that are in contact with the same side of a material under test:

$$R_s = \frac{U}{I_s}$$ [86]

The conductivities of the samples were calculated based on the following equations:

Superficial resistivity ρ_s, in Ohm per square (Ω/sq), is determined through the following expression:

$$\rho_S = \mathrm{R}\frac{D}{L}$$ [86]

Where R is the resistivity (Ω), D is the width of electrodes (cm) and L is the length between electrodes (cm).

Volumetric resistivity ρ_v, in Ohm per cm (Ω/cm), was achieved by the following equation:

$$\rho_V = \mathrm{R}\frac{A}{L}$$ [87]

Where R is the resistivity (Ω), A is the electrodes area (cm^2) and L is the distance between electrodes (cm), corresponding to the thickness of the sample. The thickness of the sample was measured using a micrometer from Fischer.

The electrical conductivity of the samples was measured at room temperature using a Keithley 487 Picoammeter/Voltage source. To minimize the effects of electric fields from neighborhood the samples were introduced in a Faraday cage (Figure 16). Two different apparatuses were used: one for measuring superficial conductivity (Figure 16 a)) and other one for the volumetric conductivity (Figure 16 b)). A voltage source was connected to the electrodes, applying a voltage across the sample. The amount of current flowing through the sample was measured by a picoammeter. For all the samples, the conductivity was measured in three different points of the sample.

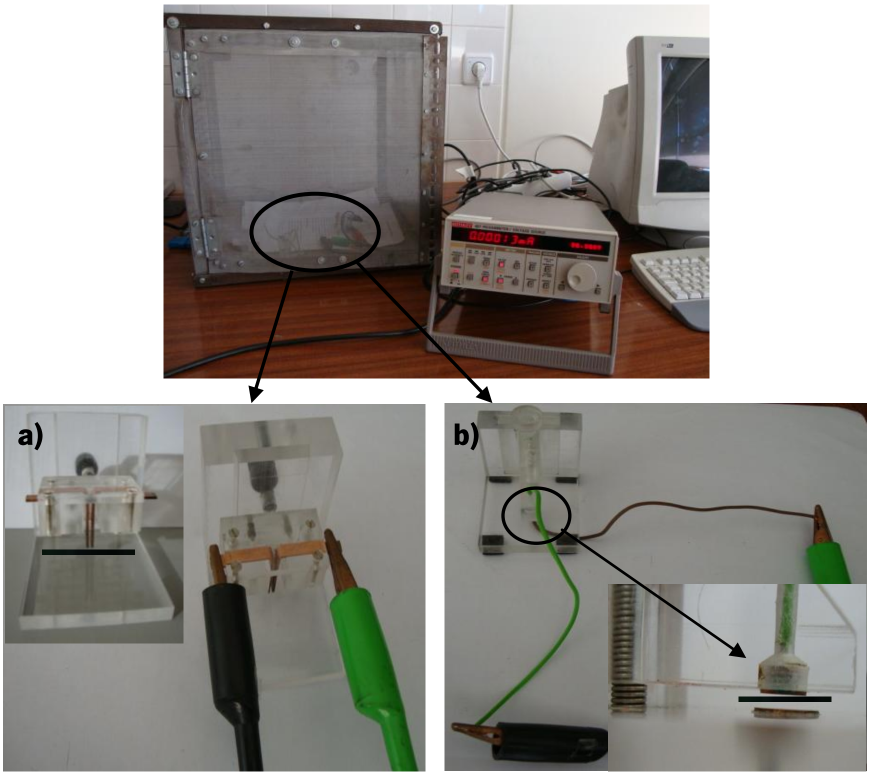

Figure 16 – Apparatus for measure electrical conductivity: a) superficial conductivity and b) volumetric conductivity.

2.2. Fourier-Transformed Infrared Spectroscopy

In order to complement the characterization studies the Fourier-transformed infrared (FTIR) spectroscopy was employed to confirm the presence of PANI on the BC discs, reflecting the effectiveness of polymerization reaction.

Fourier transformed infrared spectrometer-attenuated total reflectance (FTIR-ATR) spectra for the BC discs were recorded with a Perkin-Elmer Spectrum One IR spectrometer, within the range between 4000 and 750 cm^{-1}.

All spectra were recorded at room temperature at the resolution of 8 cm^{-1} and 32 times scanning. The software used for the data acquisition was IRSolution 1.20 Shimadzu, Japan.

2.3. Morphological Characterization

Stereo Microscope and SEM were used to study the morphology of the surface of the BC composites.

For microscopic image acquisition of cross section of BC and the BC-graft-PANI composite was used a Stereo Microscope *Olympus* (Model SZ-CT) and a monochromatic camera SONY (Model CCD).

The developed BC-grafted-PANI discs were also characterized using scanning electron microscopy of Ultra high resolution, EDAX- Pegasus X4M (Figure 17). The software used for the data acquisition was XT Microscope Server Control, version 1.3.3 (FEI company). For SEM studies, the samples were mounted on metal stubs using double-sided adhesive tape and pre-coated with gold using a sputter coater.

The working principle of scanning electron microscopy (SEM) consists of examining the structure by bombarding the specimen with a scanning beam of electrons and then collecting slow moving secondary electrons that the specimen generates. SEM is typically used to examine the external structure of objects that are as varied as biological specimens, rocks, metals, ceramics and almost anything that can be observed in a dissecting light microscope.

Figure17 - Scanning Electron Microscope of ultra high resolution.

2.4. Thermal Analysis

Thermal gravimetric analysis (TGA) was performed using a thermalgravimetric analyzer (Shimadzu, TGA-50) on about 5 mg samples. The samples were heated in open alumina pans over 25°C -500°C at a heating rate of 5°C/min under air flow.

Differential scanning calorimetry (DSC) measurements of the conductive polymer samples were carried out using Shimadzu DCS-50 at temperatures ranging from 25°C to 500°C at a heating rate of 5°C/min.

Acquisition of the results was done by TA-50WS software (version 1.14). For the assays, 5 mg were weighted (the exact masses were recorded) into aluminium pans (from Izasa, S.A., Portugal). Open pans were used for the assays.

2.5. Viability Assay - MTS Test

The fibroblast cell line (3T3) was cultured in Dulbecco's Modified Eagles's Medium (DMEM) supplemented with 10% calf serum and 1% penicillin/streptomycin and maintained at 37°C in a humidified incubator containing 5% CO_2. They were fed every 2 days and sub-cultured once they reached 70-90% confluence, by treatment with a trypsin/EDTA solution.

On the day of the experiment, cells were seeded (2×10^5 cells/ml) on the top of polyaniline-treated or non-treated bacterial discs previously placed on the bottom of 96-well plates. The effect of the different treatments on cell viability was assessed after 24, 48 and 96 h by using the Cell Titer 96®Aqueous ONE Solution Reagent (MTS [3-(4,5- dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium] colorimetric assay (Promega, Madison, EUA), according to the instructions provided by the manufacturer. Briefly, 100 µl from each well was transferred to clean wells and the absorbance read at 490 nm. Results were expressed relative to t = 0h, which was considered 100% of cell proliferation.

3. Results and Discussion

3.1. Production of Bacterial Cellulose-graft-Polianiline Composites

The nanoporous BC membranes/discs, used to producee the BC-graft-PANI composite were successfully synthesized by fermentation of *G. xylinus* in *HS* medium. Figure 18 presents images of the obtained BC. The dry and hydrated BC discs had a thickness of 1mm and 5mm, respectively.

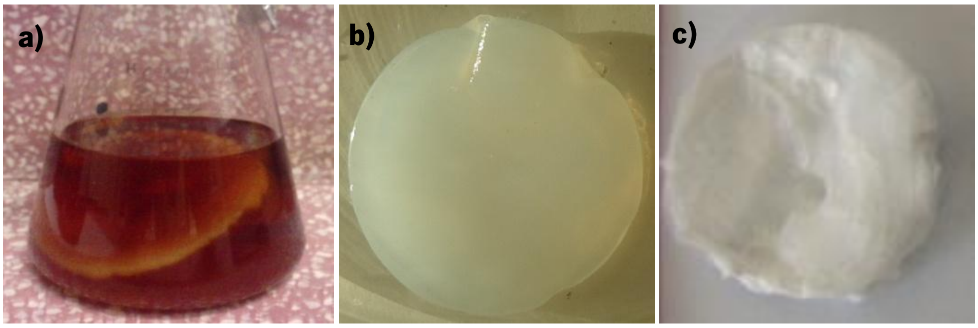

Figure 18 – Bacterial Cellulose: a) in the medium culture; b) wet BC discs and c) freeze-dried BC discs.

Standard PANI, used as control, was produced and obtained in the form of a dark green powder (Figure 19). PANI was kept in desiccators at room temperature.

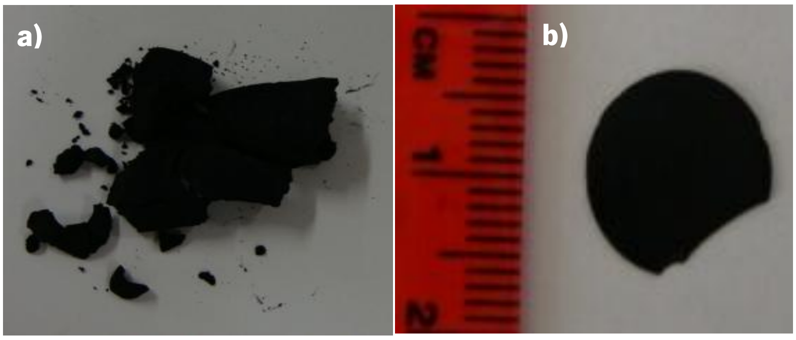

Figure 19 – PANI powder and PANI disc (obtained after compressing).

3.1.1. Direct Polymerization Method

On this method, the *in situ* polymerization of aniline in the presence of hydrated BC discs in acidic aqueous media was performed using ammonium peroxydisulfate (APS) as oxidant and CB-graft-PANI was obtained as the protonated emeraldine salt form (conducting PANI) (Figure 20).

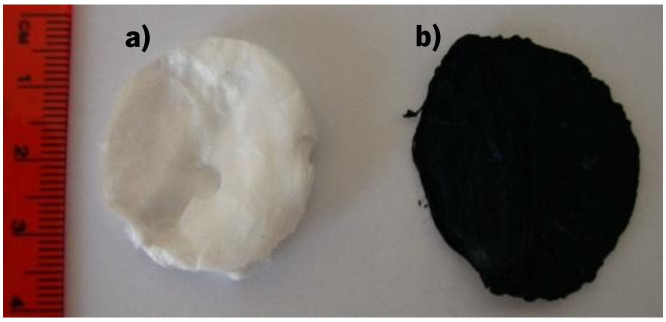

Figure 20 – BC discs a) before and b) after aniline polymerization.

It was expected that the hydroxyl groups of BC would interact with amine groups of aniline to form the hydrogen bands which ensure the uniform distribution of aniline on the surface of BC nanofibers and then, after adding the oxidant solution, the aniline monomer would polymerize in the BC network [9].

Comparing both pictures, Figure 20 a) and b), the composite discs showed a dark green color which suggests the PANI formation at the surface of the BC nanofibers.

Figure 21 shows the microscopic images of cross section of BC and the BC-graft-PANI composite (1:10 (6h)). As can be observed in figure 21 b) the polymerization occurs mainly at outer regions of BC, forming a gradient along the BC network, with lower amount of PANI diffused within the disc. This can be explained as follows: once the polymerization starts at the surface of the BC (noticeable by the formation of green points on the BC at the beginning of polymerization), it hinders the spread of PANI inside the BC matrix, forming a gradient along the BC.

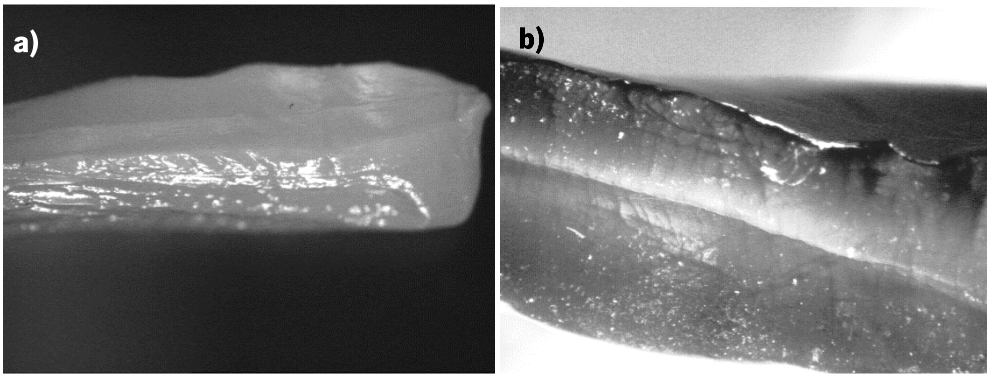

Figure 21 – Microscopic images of a) native BC and b) BC-graft-PANI composites.

Figure 22 show different samples produced by this method. Varying the monomer concentration and the polymerization time produces different shades of green, becoming darker with the increase in these two parameters. With high concentration of aniline the differences in color samples after 6 hours of polymerization is not significant.

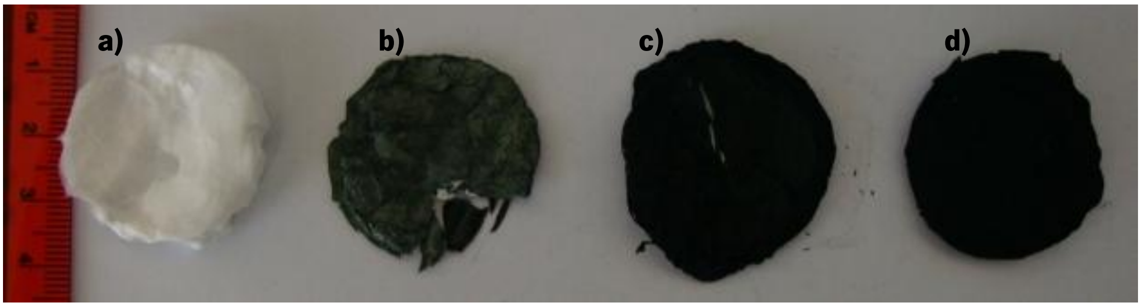

Figure 22 - Standard BC discs and CB-graft-PANI discs after a polymerization time of 6 hours: b) 1:1; c) 1:5 and d) 1:10

3.1.2. Surface Modification Method

Figure 23 shows some samples produced by this method. The samples do not display the dark green color typical of conductive PANI.

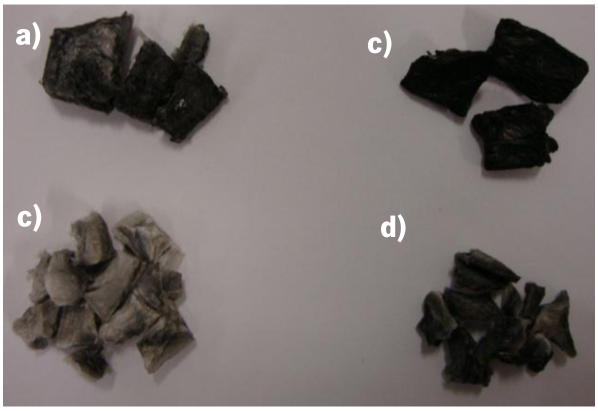

Figure 23 – BC membranes: a) A2hP12h, b) A2hP24h, c) A4hP12h and d) A4hP12h.

3.1.3. Sulfonation Method

Figure 24 shows the different samples produced by oxidation and further sulfonation of BC discs. In method a (Figure 24 a) there is some dark green coloration although much lower when compared with the direct polymerization method. Using method b the color of the samples suggests that there is no formation of conductive PANI (emeraldine salt) in the surface of BC discs.

Figure 24 – Results of BC discs by means of the sulfonation method: a) 3^{rd} step without washing (a)) and b) 3^{rd} step with washing (b)).

3.2. Weight Loss Measurements

In table 6 shows the efficiency (%) of polymerized BC discs by means of *in situ* oxidative polymerization. An increase of the monomer concentration leads to an increase in the weight of the sample, which in turn suggests the successful incorporation of PANI in BC discs.

Table 6 – Efficiency of BC-graft-PANI samples by means of *in situ* direct polymerization.

Ratio CB:Aniline	Polymerization time (h)	Weight loss (%)	Weight increase (%)
1:1	6	3.6	-
1:5	3	2.18	-
	6		8.8
	9		26.29
	12		2.44
	24		10.24
1:10	3		46.85
	6		104.55
	9		42.75
	12		33.44
	24		31.02

The study of efficiency of BC-graft-PANI produced by surface modification method can be seen in Table 7.

Table 7 – Efficiency of BC composites for the different activation and polymerization times.

Activation time (hours)	Polymerization time (hours)	Weight loss (%)
2	2	29.20
	6	51.05
	12	42.15
	24	22.61
4	2	60.89
	6	59.37
	12	33.30
	24	32.77

The considerable weight loss can be explained by the use of membranes instead of discs. These membranes were cut into pieces (Figure 23), which after stirring resulted in loss of material to the solution (formation of small fragments), leading to a decrease in final sample weight.

The direct polymerization method proved to be more efficient when comparing to the surface modification method as shown in Figure 25.

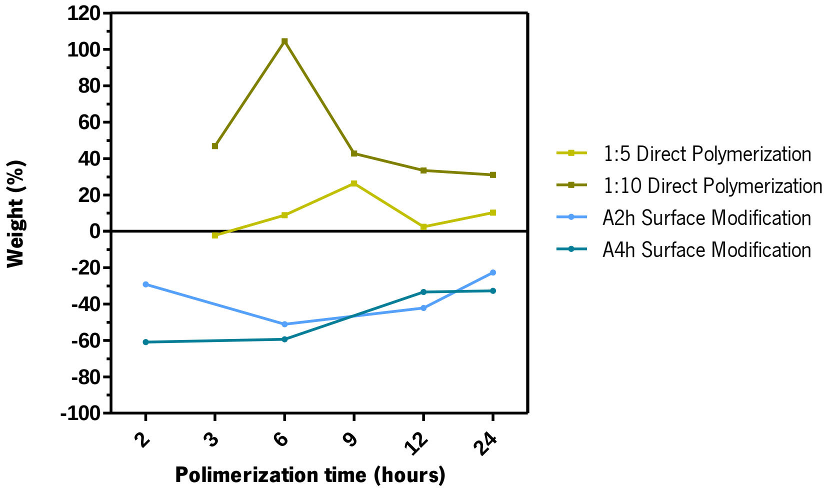

Figure 25 – Efficiency of polymerization (%) of BC discs produced by means of direct polymerization and by the surface modification method.

The weight loss of the samples produced by means of the sulfonation method was in the range of 42-64%. The oxidation and sulfonation of BC discs represents a higher weight loss. This occurs mainly in the oxidation step, where there was a sharp reduction in size of the discs.

This method proved to be ineffective, due the fact that it´s time consuming and results in high weight loss, together with the weak coloration of the produced samples, suggesting the low PANI formation on BC discs and consequently weak conductivity.

3.3. Electrical Behavior

Conductivity is a measure of electrical conduction and thus a measure of the ability of a material to pass a current. Generally, materials with conductivities less than 10^{-8} S/cm are considered insulators, materials with conductivities between 10^{-8} and 10^{3} S/cm are considered similar to semiconductors, and materials with conductivities higher than 10^{3} S/cm are considered conductors [4].

The electrical conductivity measurements were carried out on CB-graft-PANI by applying a voltage across the sample, causing a current flowing through the sample measured by an ammeter. The calculated electrical conductivity for BC, was $7.5*10^{-11}$ S/cm. For standard PANI the best result was $2.84*10^{-3}$ S/cm as shown in Figure 26.

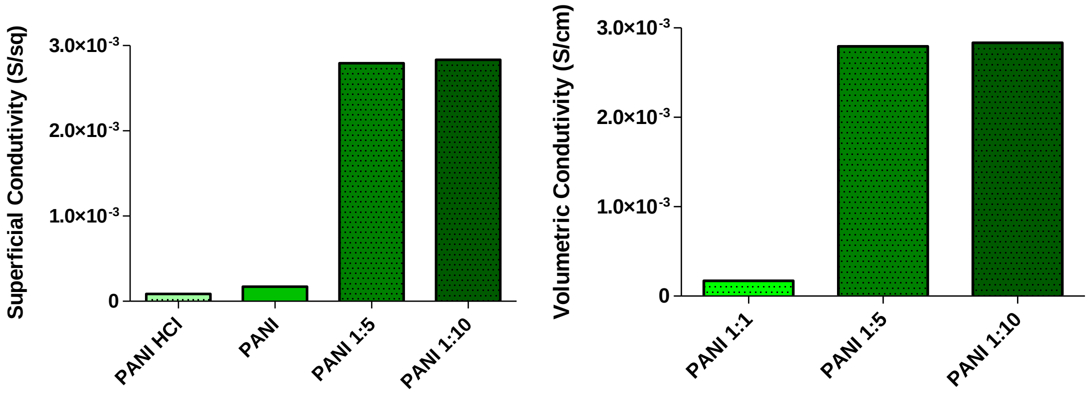

Figure 26 – Superficial/Volumetric conductivity of different PANI.

The increase in electrical conductivity of CB discs produced by means of the *direct in situ* polymerization method is considerable and can be observed in Figures 27-31. These values of electrical conductivity are in the range of values for a semiconductive material (10^{-8} and 10^{3} S/cm) [4].

As mentioned above, the electrical conductivity of PANI depends on several issues and monomer concentration is one of them, which can be demonstrated in Figure 27. An increase in the amount of aniline in the polymerization reaction leads to an increase in conductivity of the samples.

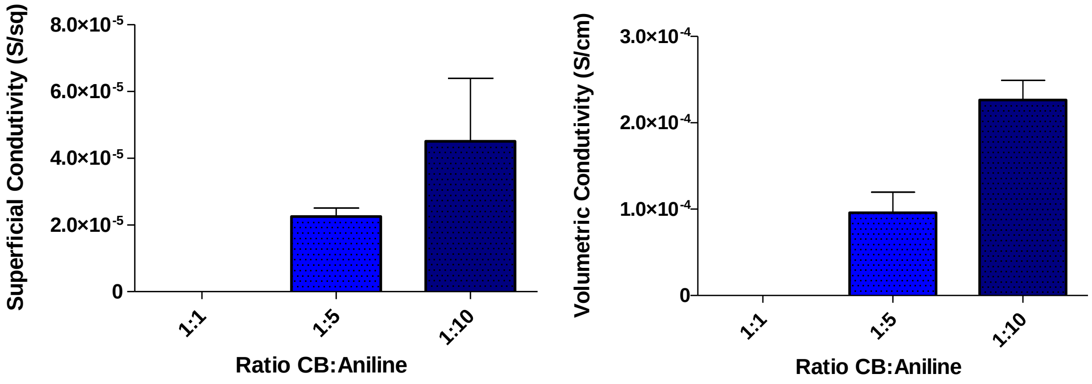

Figure 27 – Superficial and volumetric conductivity for different ratios of CB:Aniline for a polymerization time of 6hours.

Figure 28, 29 shows the effects of reaction times on the electrical conductivity of BC-graft-PANI composites. Figure 28 illustrates the conductivity for a CB:aniline ratio of 1:5. The best results were obtained at 6 hours, 2.25*10^{-5} S/sq and 9.6*10^{-5} S/cm, and 12 hours with an electrical conductivity of 2.25*10^{-5} S/sq and 9.2*10^{-5} S/cm.

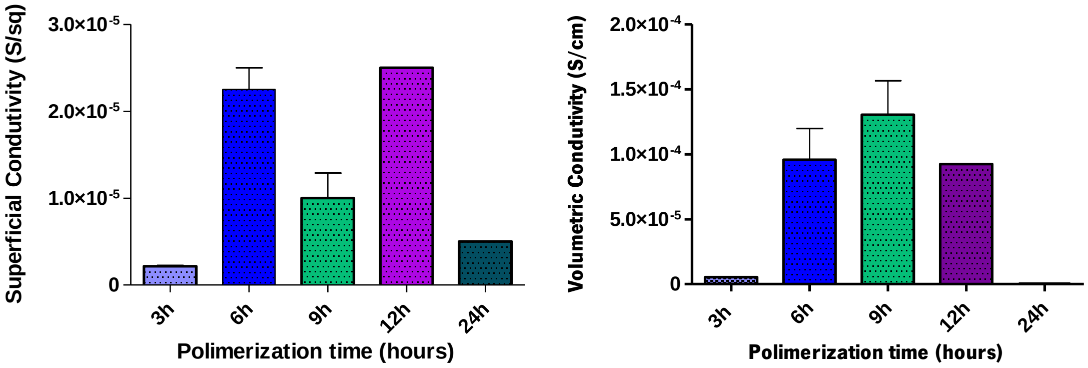

Figure 28– Effects of polymerization time on superficial and volumetric electrical conductivity of BC-graft –PANI for a CB:aniline ratio of 1:5.

For the ratio 1:10, the best conductivity was found at 6 hours and 12 hours for superficial conductivity 4.5*10e^{-5} S/sq and 6.7*10e^{-5} S/sq respectively; and for volumetric conductivity the best results were 2.26*10e^{-4} S/cm for 6 hours and 1.56*10e^{-4} S/cm for 9 hours (Figure 29).

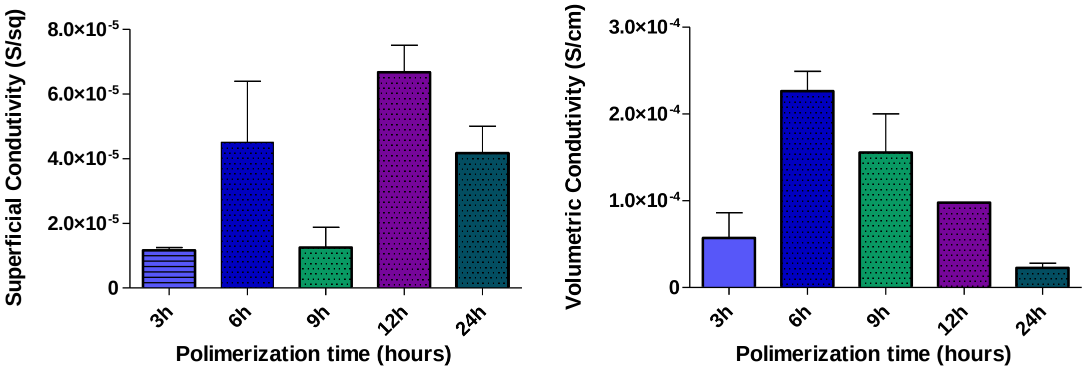

Figure 29– Effects of polymerization time on superficial and volumetric electrical conductivity of BC-graft –PANI for a CB:aniline ratio of 1:10.

The results suggested that the time of polymerization does not have a pronounce role in terms of conductivity. It was observed with increasing reaction time, there is an increase in the amount of PANI deposited on the surface of BC (3 hours to 6 hours of time reaction). This initial increase can be justified as a result of the fact that disconnected PANI nanoparticules gradually grow into a continuous nano-coating, covering the BC disc. However, excessive polymerization times, lead to the precipitating or aggregating of PANI particles and excessive oxidation of the monomer with the fracture of the PANI conjugated chain, as suggested by Hu *et al* [9], causing a decrease in electrical conductivity.

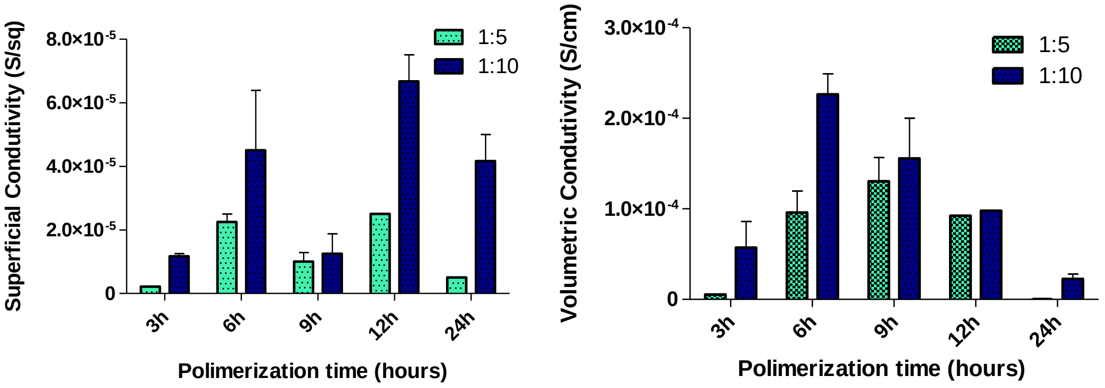

Figure 30 – Superficial and volumetric conductivity: balance between 1:5 and 1:10 CB:aniline ratio for different polymerization times.

One more time comparing both 1:5 and 1:10 for different times of polymerization (Figure 30), leads to the conclusion that, as expected, more concentration of aniline monomer results in higher conductivities

The obtained results are in concordance with values found in literature (Table 3). However, in some papers is possible to find higher values of conductivity. This can be related with the use of higher concentrations of aniline and, as reported by Mo *et al* [17], PANI content increased with the amount of aniline.

Therefore, the BC-graft-PANI composites produced by means of the *direct in situ* polymerization method have been successfully synthesized and the best electrical conductivities results were for a monomer concentration ratio of 1:10 and a polymerization time of 6 hours, $2.26*10^{-4}$ S/cm.

For evaluating the electrical behavior of the BC-graft-PANI composites produced by means of sulfonation method only the samples that were expected to have more conductivity were measured. The measured conductivity showed that the samples prepared by this method are insulators having very low electrical conductivity in the range of $1.33*10^{-09}$ to $2.50*10^{-09}$. This may have happened because, despite the use of aniline hydrochloride, as the reaction occurred in a distilled water medium, the pH of the reaction probably was not sufficiently low for the formation of PANI emeraldine.

Since the BC composites produced by surface modification and sulfonation methods had a much less dark green color, typical from conducting PANI. Consequently, lower electrical conductivities, together with large weight loss, it can be concluded that the direct *in situ* polymerization process was the most simple and effective.

The characterization of cellulose-graft-polyaniline discs focused on the best conditions obtained by means of the *in situ* chemical oxidation of aniline in BC hydrated discs.

3.4. Fourier-Transformed Infrared Spectra

The vibrational spectrum of a molecule is considered to be a unique physical property and is characteristic of the molecule. The infrared spectrum is formed as a consequence of the absorption of electromagnetic radiation at frequencies that correlate to the vibration of specific sets of chemical bonds from within a molecule. The fundamental requirement for infrared activity, leading to absorption of infrared radiation, is that there must be a net change in dipole moment during the vibration for the molecule or the functional group under study. While it was stated that the fundamental infrared absorption frequencies are not the only component to be evaluated in a spectral interpretation, they are the essence and foundation of the art [88].

In order characterize the chemical structure of CB-graft-PANI composites, Fourier transform infrared (FTIR) spectroscopy was used and FTIR spectra are shown in Figures 31-33.

The infrared spectrum of natural BC and standard PANI, used as control, are shown in Figure 31.

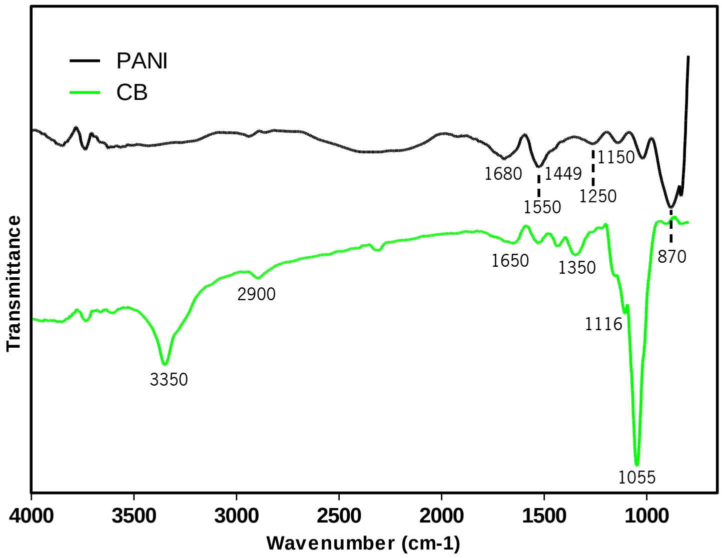

Figure 31 - FTIR spectra of (a) CB discs and (b) standard PANI.

The FTIR spectrum of pure CB displays a strong peak around 3350 cm^{-1}, corresponding to the stretching vibration of hydroxyl groups. The peak at approximately 1350 cm^{-1} is attributed to the O-H bending. The absorption band, at approximately at 2900 and 1650 cm^{-1} were assigned to the C-H stretching in pyranoid ring and the H-OH bending of the absorbed water, respectively. The peak at approximately 1116 cm^{-1} arises from the C-O bond`s asymmetric bridge stretching. The main peak in the spectrum, at 1055 cm^{-1}, corresponds to the C-O-C pyranose ring skeletal vibration.

The typical feature of PAN FTIR-ATR spectroscopy is well known in literature [89]. The PANI spectrum (Figure 31) is characterized by peaks at approximately 1550 and 1449 cm^{-1} due to the quinine and benzene ring stretching vibration, respectively. The band at about 1680 cm^{-1} can be assigned to the C=O stretching of carbonyl group of the ring. The absorption bands around 1250 and 1150 cm^{-1} are assigned to stretching vibration of the C-N band and to the aromatic C-H stretching vibration. The band at 870 cm^{-1} corresponds to the out-of-plane bending vibration of the C-H band of 1,4 - disubstituted benzene ring.

In Figure 32 the variation of molar ratio (CB:aniline) for 6 hours of polymerization can be observed.

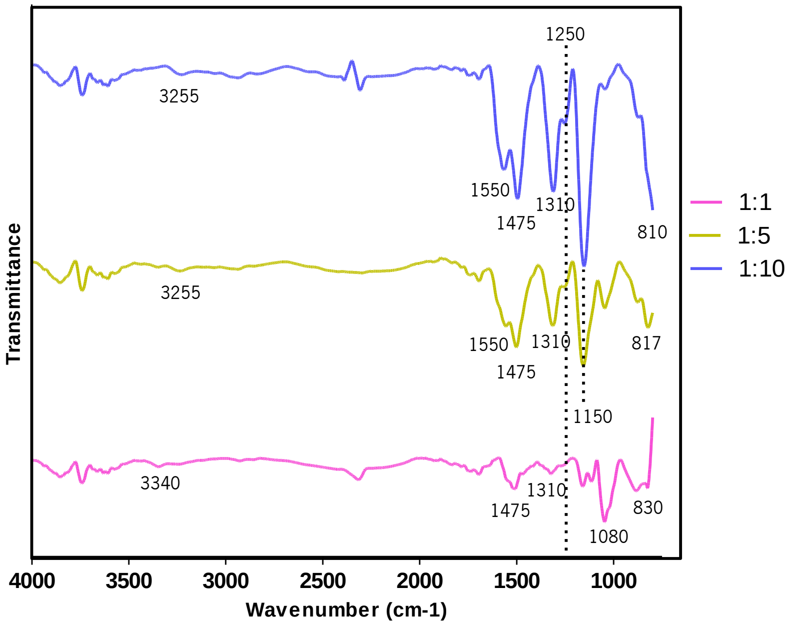

Figure 32 - FTIR spectra obtained after 6 hours of polymerization and varying the aniline monomer concentration.

The successful incorporation of PANI in BC nanofibers was confirmed by FTIR. The spectra of CB-graft-PANI composites display the main characteristic absorptions of BC and PANI. However, some particularities can be observed. Comparing them with the spectrum of pure BC, it was found that the typical absorption peak of stretching vibration of OH group at 3350 cm^{-1} was shifted to approximately 3340 cm^{-1} and the peak almost disappear. According to Hu *et al* [9], this suggests that the BC composites were successfully produced and the intermolecular hydrogen bands are broken and more hydrogen groups became accessible which helps to form the uniform dispersion of PANI in the BC discs. Moreover, the bands at approximately 1310 and 1250 cm^{-1} characteristics of conducting proponated PANI, are well distinguished in the spectrum of BC composites.

Figure 33 shows the effect of the reaction time on the amount of PANI in the CB-graft-PANI discs.

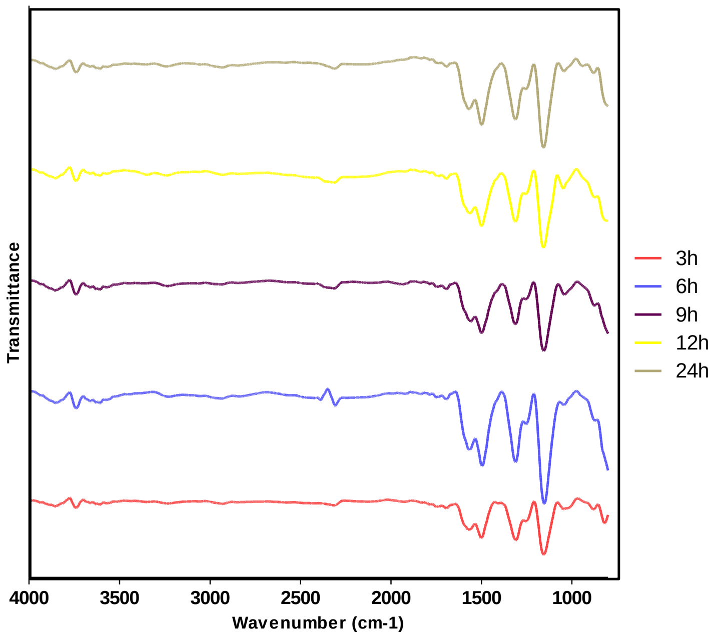

Figure 33 – FTIR spectrum of CB-graft-PANI composites with a ratio 1:10 and different times of polymerization reaction.

The peak`s intensity became stronger with increasing reaction time and consequently the content of PANI on the BC discs. This effect is especial noticeable from 3 hours to 6 hours of time reaction. After this polymerization time, the changes are not significant.

Thus, the similarity between CB-grafted-PANI and pure PANI powder suggests, one more time, that BC nanofibers were successfully coated with PANI, as already reveled by the conductivity results. However to confirm the grafting of PANI on BC, further analysis should be performed, such as NMR or mass spectroscopy.

3.5. Morphological Characterization

Scanning electron microscopy (SEM) enabled the characterization of the morphology of the prepared BC-graft-PANI in terms of shape, matrix and roughness. Typical SEM micrographs of the BC are shown in Figure 34.

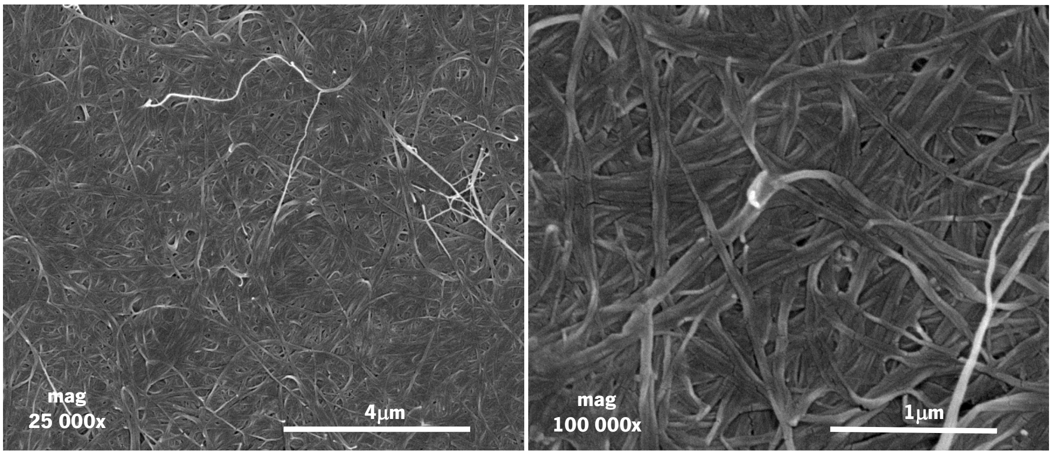

Figure 34 - SEM micrographs of BC discs used as control.

The SEM micrographs allow visualization of the ultrafine network structure of BC disc, consisting of nanofibers with a diameter ranging from 40nm to 70nm. The BC nanofibers present near-cylindrical shape and a smooth surface.

The typical morphology of the developed BC-graft-PANI composite is shown in Figure 35, 36.

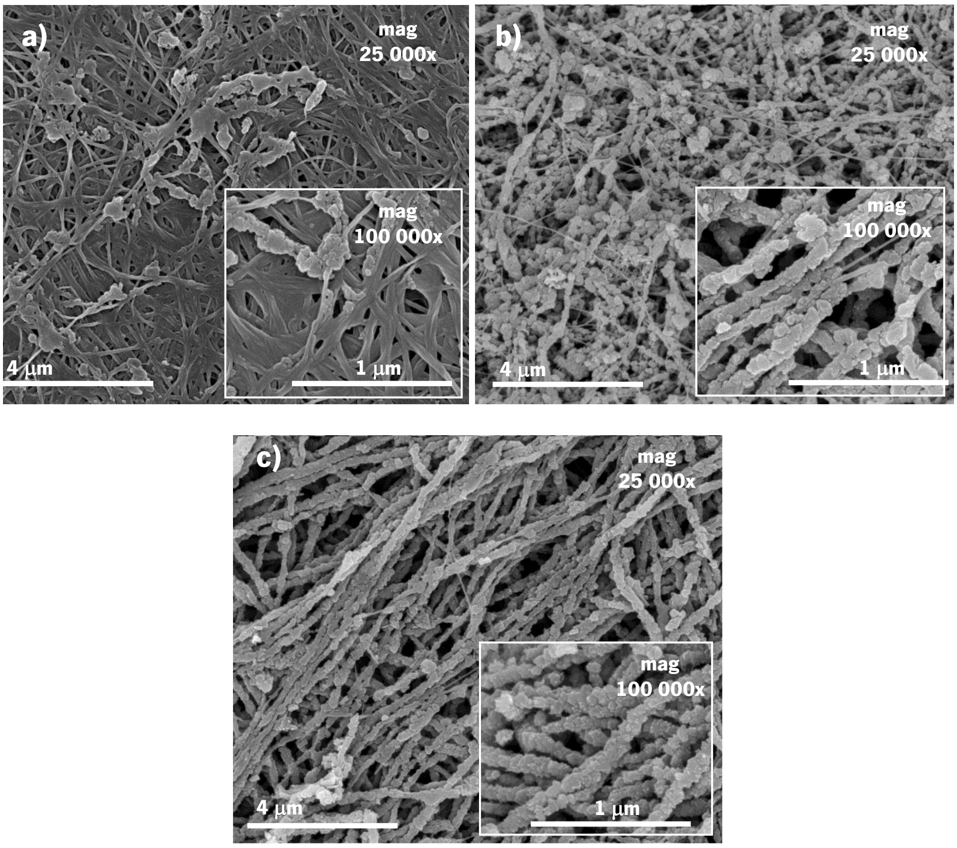

Figure 35 - SEM micrographs of a) 1:1 (6h), b) 1:5 (6h) and c) 1:10 (6h).

In CB composite micrographs, it is possible to observe PANI in the matrix conferring different degrees of roughness (Figure 35), increasing with the amount of PANI on the surface.

As shown in Figure 35, the PANI on the surface of BC shows a similar nano-granular morphology for all the samples, typically of PANI coatings [90]. In Figure 35 (a) is also possible to observe some PANI islands, whereas in figure 35 (c) the surface of BC was completely covered with PANI.

Thus, comparing the polymerized discs with different monomer concentrations (1:1, 1:5, 1:10) showed that the increase of this parameter leads to differences in the morphology of the samples. The higher amount of aniline in 1:10 discs, leads to a higher adhesion of PANI in the BC, than in the 1:5 and 1:1. This is also true when increasing of polymerization time, as more PANI is incorporated on BC discs (Figure 36).

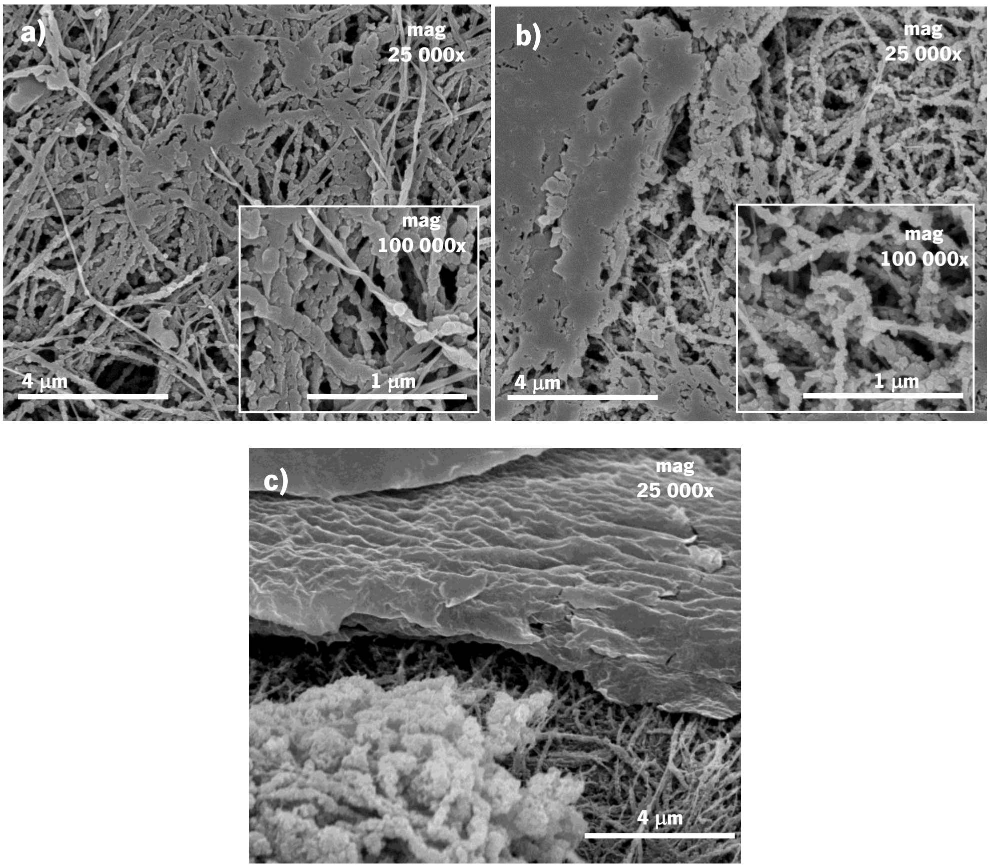

Figure 36 - SEM micrographs of a) 1:5 (12h) and b) 1:10 (12h); and c) 1:10 (12h).

The 1:10 (12h) reaction conditions present the highest amount of PANI at the surface of BC discs.

As shown in the SEM microphotographs (Figures 35, 36), many pores with a diameter of about 200 nm were observed on the surface of all the samples. For higher polymerization reaction time (12hours) this porosity started to decrease. For high polymerization times, the PANI nano-granular particles aggregated to the BC fibbers, merged to form a continuous nano-layer along the BC surface (Figure 36 c).

Therefore, the prepared samples seem to be structures with very good electrical conductivity due to the high amount of PANI presented on the surface of BC. This results are in concordance with others found in literature [8, 9, 12, 71, 74].

However, the processing methodologies should be improved in order to increase the porosity, the pores interconnectivity and pores size, to be more favorable for cell attachment for new nerve tissue in growth.

3.6. Thermal Analysis

The thermogravimetric analysis (TGA) measures the mass change of a substance as function of temperature, while the substance is subjected to a controlled temperature programme and differential scanning calorimetry (DSC) provides information about thermal changes that do not involve a change in sample mass.

To assess some of the physicochemical properties changes and thermal stability, the thermal properties of BC-graft-PANI were investigated and are shown in Figures 37, 38. The TG and DSC curves of pure BC and PANI are also shown for comparison.

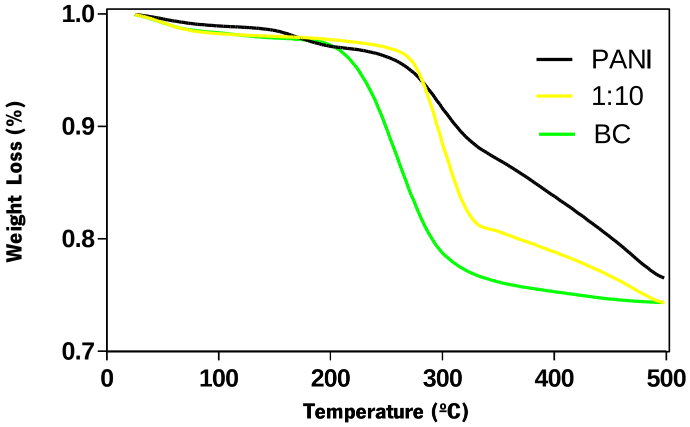

Figure 37 - TGA Thermogram of BC, PANI and BC-graft PANI.

As can be observed in Figure 37 the TG curve of BC-graft-PANI (1:10 12h) shows that weight loss occurred in three different stages. During the initial stage from room temperature to 100°C there is a weight loss assigned to the evaporation of the water present in the composite. From 210° to 340°C the samples undergo a strong weight loss, which can be explained by the burning of BC composite. The final step in weight loss from 340 to 500°C can be attributed to the thermal-oxidative degradation of PANI.

When comparing with the TG of pure CB, it can be seen that BC also experiences a similar weight loss process, although there are some differences. The onset temperature of the thermo-oxidative degradation of BC discs is lower on pure BC than on BC-graft-PANI and a more gradual weight loss over the wide temperature range of the composite, indicating that the thermal stability of the composites is larger than that of BC. Moreover, the highest weight loss of the BC happens at higher temperatures than in CB-graft-PANI and in CB discs a small weight loss is noticeable even at temperatures exceeding 450°C, which can be associated with the weakened inter and intramolecular hydrogen bonding of BC in the CB-graft-PANI discs and the absence of intermolecular hydrogen bands of CB, respectively.

The thermal stability of BC is increased by the incorporation of PANI and increased by increasing the polymerization time. This thermal behavior is in concordance with results obtained by Mo *et al.* [17], Stejskal *et al.* [72] and Hu *et al.* [9].

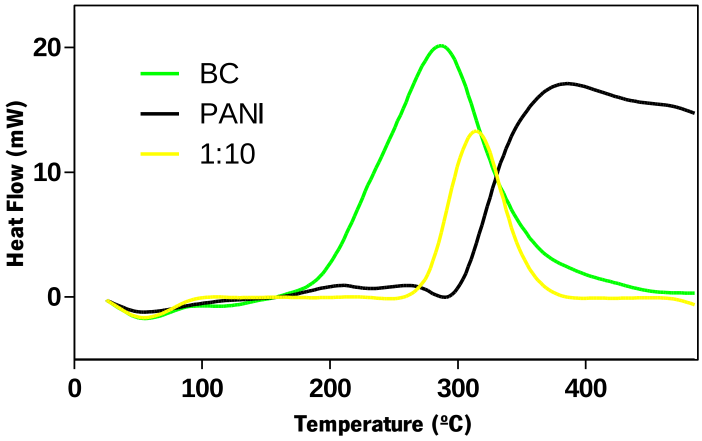

Figure 38 - DCS curves of BC, PANI and BC-graft PANI.

Figure 38 (c) shows DCS termograms of BC-graft-PANI composites, and it revealed an endothermic peak from 0 to 100°C attributed to the removal of the loosely bound water molecules present in the composite matrix. The exothermic peak at approximately 312°C can be assigned to interchain crosslink and thermally effected morphology changes [8]. This exothermic peak is larger in CB than in the composite which confirms the thermal stability of the resulting CB-grat-PANI. The thermogram confirms also the absence of glass transition (T_g) and melting temperature (T_m) for all analysis

3.7. MTS Assays

In order to evaluate the cell viability, MTS test was performed. For that, 3T3 cells were used.

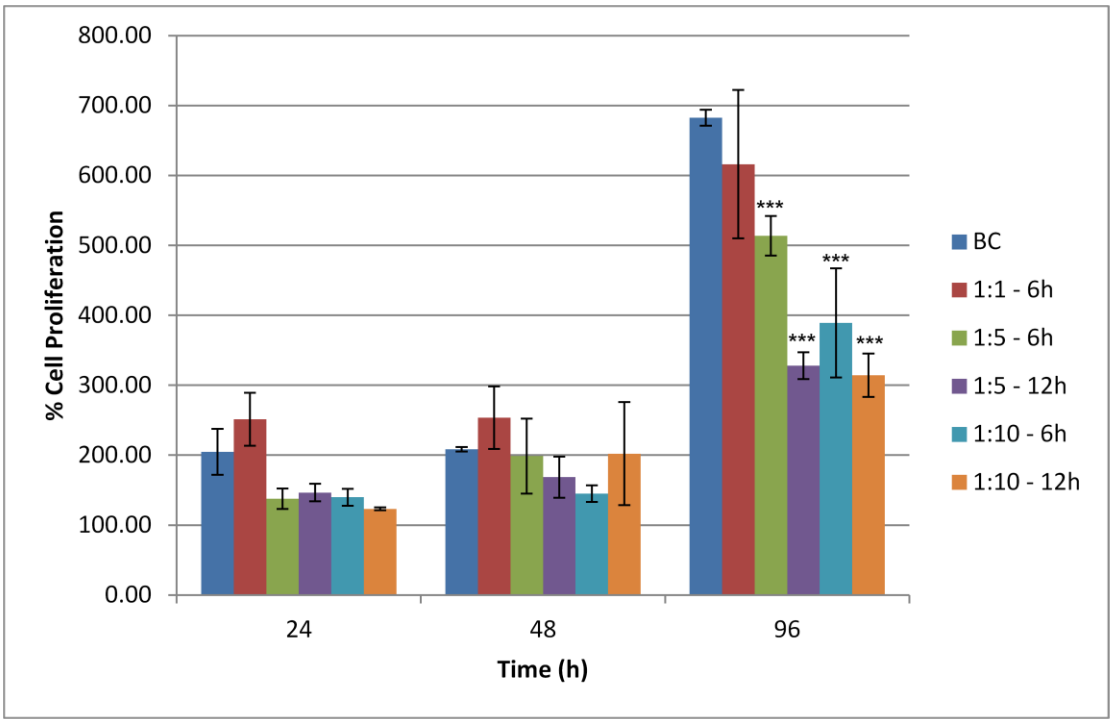

Figure 39 – Cell proliferation assessed by MTS assay. *** $p < 0.001$, relative to non-treated BC, for the same timepoint.

As can be observed from Figure 39, none of the treatments were toxic to the cells, as none of them resulted in a decrease of cell viability to values below the initial ones (considered as 100%). Moreover, none of the PANI-treated discs affected cell proliferation up to 48h. However, at 96h, only the 1:1 (6h) sample did not affect cell proliferation. All the others induced a statistically significant decrease of this parameter.

CHAPTER IV.

CONCLUSION AND FUTURE WORK

Conclusions and Future Work

Conductive BC-graft-PANI composites materials have been successfully produced in situ by oxidative polymerization of aniline using hydrated BC as the template.

The electrical conductivity increased from 7.5*10^{-11} S/cm to 2.26*10^{-4} S/cm by controlling the time of polymerization and the molar Ratio of CB:aniline. Under the assayed experimental conditions, the optimum grafting efficiency was find at a CB:aniline ratio of 1:10 and with a time reaction of 6 hours.

FTIR spectra indicated that BC nanofibers were successfully coated with PANI. In addition, SEM shows that this process produced electrically conductive BC films containing well-dispersed PANI, with a granular structure with grain sizes ranging from 100 to 200nm. After 12h of polymerization, the PANI particles deposited on the surface of BC, merged to form a continuous nano-layer by taking along the BC template. The modified nanocellulose showed increased thermal stability and according to MTS test none of the optimized samples were toxic to the cells.

Based on the above observations, the CB-graft-PANI biopolymer combines good processability with improved electrical properties, higher thermal stability and biocompatibility which could be applied in chemical and biosensors, optical and electrical displays and for biomedical applications.

Future work will, mandatorily, lead to the study and optimization of other reaction parameters, which could interfere in the mechanism of the BC-graft-PANI, such as: temperature, pH, use of different oxidant agents, and the use of higher amounts of aniline.

Other very important issue is the characterization of the mechanical properties of conducting BC.

For biomedical applications, necessary improvement of the processing methodologies is required, in order to increase the porosity, the pores interconnectivity and pores size, to be more favourable for cell attachment for nerve tissue in growth. Also, the study of the ability of these materials in promoting cell adhesion and ingrowth is essential, in order to confirm their potential for tissue nerve regeneration.

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