In: Cellulose: Structure and Properties… ISBN: 978-1-60876-388-7
Editors: A. Lejeune, T. Deprez, pp. 427-458

Chapter 18

BACTERIAL CELLULOSE: PROPERTIES, PRODUCTION AND APPLICATIONS

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ABSTRACT

Bacterial cellulose (BC) is an outstanding biomaterial with unique properties, including high water holding capacity, high crystallinity, ultrafine fiber network, high tensile strength in the wet state and the possibility to be shaped into three-dimensional (3D) structures during synthesis. These properties support a wide range of applications, in human and veterinary medicine, odonthology, pharmaceutical industry, acoustic and filter membranes, biotechnological devices and in the food and paper industry.

BC is considered biocompatible, presenting an insignificant foreign body reaction; it does not elicit chronic inflammatory responses *in vivo*. These properties make BC very interesting for tissue engineering applications. Indeed, ongoing research of BC products includes a wide range of biomedical applications: treatment of chronic wounds and burns as temporary coverage, artificial cardiovascular tissues, and guided regeneration of bone, cartilage and nerve.

INTRODUCTION

Bacterial cellulose is produced by bacterial strains from the genera *Acetobacter*, *Agrobacterium*, *Pseudomonas*, *Rhizobium* and *Sarcina*, the last one being the only genus of Gram-positive bacteria in this field [1]. Interestingly, only a few bacterial species, taxonomically related to this genus, extracellularly secrete the synthesized cellulose as fibers.

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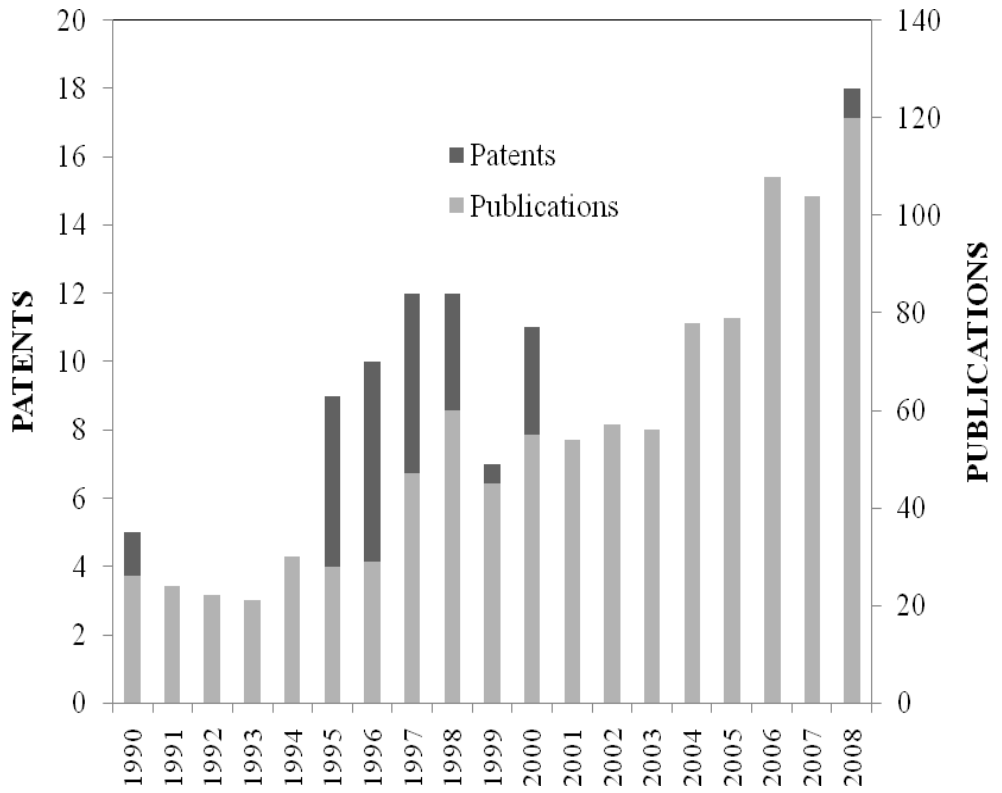

Figure1. Publications and patents on bacterial cellulose.

While the first studies on BC were geared towards elucidating the cellulose biosynthetic pathway, BC has quickly developed into a field of study of its own, as observed by the growing number of patents and publications worldwide (Figure 1). Special attention was given to strains from *Gluconacetobacter xylinus* (=*Acetobacter xylinum*), first described by Brown in 1886 [2]. While the secreted cellulose is identical to the one produced by plants, regarding the molecular structure, it is chemically pure, *i.e.* not mixed with non-cellulosic polysaccharides [1, 3-6]. Its unique properties account for its extraordinary physico-chemical and mechanical behaviour, resulting in characteristics that are quite promising for modern medicine and biomedical research [5, 7-11].

Biosynthesis, Structure and Properties

The classical medium to culture *G. xylinus* and maximize the growth and cellulose production was described by Hestrin and Schramm. The pH of the medium is 6 and the optimum growth temperature is 30 ^{o}C, though the bacteria grow well over a temperature range of 25 to 30^{o}C. The static culture leads to the production of a cellulose pellicle holding bacterial cells floating on the surface medium. In a culture medium aerated by shaking, bacteria grow faster, but less cellulose, presented as ball-shaped particles, is produced. When *G. xylinus* is cultured on solid medium, the colonies have a dry, wrinkled appearance [12, 13].

The ultrastructure of the cellulose synthesis apparatus is best understood in *G. xylinus.* The cellulose synthase is considered the most important enzyme in the bacterial cellulose biosynthesis. The cellulose synthase operon codes protein complexes aligned along the long axis of the cell. Cellulose synthesizing complexes are present in the surface of the bacteria, next to the cell membrane pores where the cellulose fibrils are extruded through, associating with other fibrils and making up the ribbon of crystalline cellulose [1, 6]. 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 [12].

Several studies were developed to clarify the physiologic role of cellulose. As the cellulose matrix is less dense than water, it has been proposed to allow maintaining the bacterial cells in an oxygen-rich environment. Additionally, it allows protecting the bacteria from ultraviolet light, competing microorganisms and heavy-metal ions, while retaining the moisture and allowing nutrient supply by diffusion [5, 11, 13, 14].

As *Gluconacetobacter* microorganisms are mandatory aerobes, under static conditions, BC is synthesized at the air/liquid interface of the culture medium [1, 5]. Other relevant aspects for the BC production are the carbon and nitrogen sources and concentration, the pH and temperature, and the surface area of the fermentation system. All these aspects affect the cellulose production as well as the membrane properties, in static or agitated cell culture. Also, differences in the bacterial strains play an important role in the microstructure and production rate. Figure 2 shows a membrane produced by ATCC 10245 *G. xylinus* strain [1, 5, 15-19].

Besides macroscopic morphological differences, BC produced in static and agitated cultures differs also at various structural levels. While the fibril network remains the same, there are some differences in the structure of 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 [20].

Figure 2. Bacterial cellulose pellicle produced by ATCC 10245 *G. xylinus* strain in static culture (

As referred above, the bacterial and vegetable celluloses have the same molecular structure, both being built up of β(1→4)–linked D-glucose units. The degree of polymerization is however rather different, about 13000-14000 for plants and 2000-6000 for bacterial cellulose. Both celluloses are highly crystalline; differing in the arrangement of glucosyl units within the unit cells of the crystallites, and several studies suggests that these celluloses are synthesized by enzymatic complexes that differ at the molecular level. Also, this bacterial polysaccharide is secreted free of lignin, pectin, hemicelluloses and other biogenic compounds, which are associated with plant cellulose [1, 5, 21].

Morphology - The gelatinous BC membrane formed in static culture is characterized by a 3D ultrafine fibrous network structure, containing about 99% water. The randomly assembled ribbon-shaped fibrils are less than 100nm 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% [22-26]. Crystallographically, BC is a Cellulose I, with 60% Iα /40% Iβ [11, 26]. The crystallographic molecular arrangement may influence the physical properties, as the allomorphs have different crystal packing, molecular conformation, and hydrogen bonding [22, 26]. In 2006, Sanchavanakit characterized BC pellicles obtained after 48 hours culture: the surface area of the air-dried BC films was 12.6 m^2/g, with a pore size distribution ranging from 45 to 600 Å. The pore diameter of the air-dried film was inferior to 0,1 μm; however, when the air-dried pellicle was swollen with water, at 30 ^oC, the apparent pore diameter raised to 0.2-1.0μm [27]. Due to its high crystallinity and small fiber diameter, BC possess excellent mechanical strength and high surface area when compared to plant derived cellulose [28] and the application and biological function of celluloses are based on its distinct fiber morphology [22].

Mechanical properties – Both the micro and macrostructure of BC are influenced by the growing culture environment and the treatment after synthesis. According to Iguchi, a BC pellicle obtained after 7 days of culture and air-dried at 20 ^oC and low pressure, presents a Young's modulus of 16,9 GPa, tensile strength of 256 MPa and elongation of 1,7% [11]. However, when a pellicle was dried through the heat-press method described by Iguchi [29] and an excess of pressure (490 – 1960 kPa) was applied, the tensile strength as well as elongation tend to decrease, while the Young modulus remains constant. According to Sanchavanakit (2006), a BC dried film (from a 48h grown culture) with a thickness of 0.12 mm presents a tensile strength and break strain of 5.21 MPa and 3.75%, whereas for the wet films the values are 1.56 MPa and 8.00%, respectively [27]. The high Young's modulus and tensile strength of BC films seems to result from its high crystallinity, high planar orientation of ribbons pressed into a sheet, ultrafine structure, and the complex network of the ribbons [30].

Water holding capacity - BC is highly hydrophilic, holding over 100 times its weight in water. Klemm and colleagues showed that the "never dried" BC has water retention values (WR) in the range of 1000%, drastically decreasing after air-drying to values that can be compared with those of plant cellulose, 106% and 60%, respectively. The method of drying has been shown to affect the BC porosity, freeze-drying (WR of 629%) being reported as the most effective method to preserve the porous structure [5].

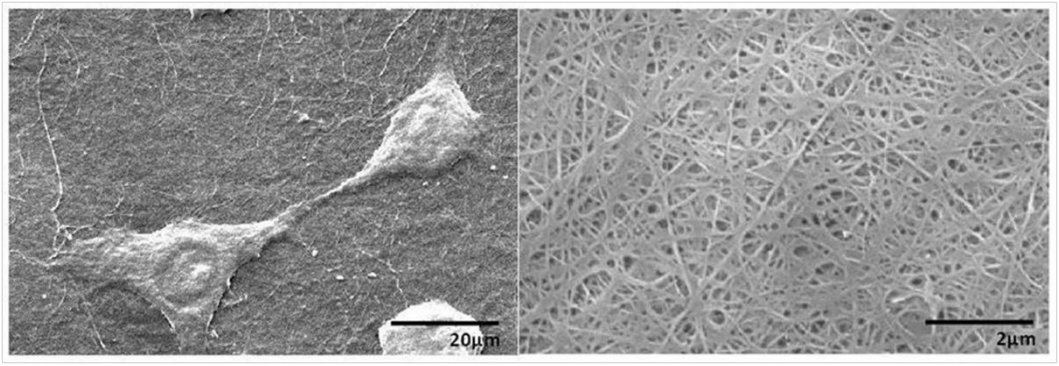

Figure 3. Scanning eletron microscopy of bacterial cellulose. Fibroblasts adhered on bacterial cellulose membranes after 24h in culture (Left, 1000x); () detail of BC membranes surface (Right, 10.000x).

Permeability – Sokolnicki *et al.* carried out mass transfer experiments to characterize the transport of biomolecules (namely vitamin B12, lysozyme and bovine serum albumin, with molecular weight of 1355 Da, 14.3 kDa and 66.3 kDa, respectively) through hydrated BC membranes. The results indicated a dual transport mechanism of the solute through the continuous water phase and cellulose matrix, with some hindrance of molecular diffusion via fiber obstruction. Also, the 94.1% membrane porosity and its morphology indicated the existence of micro-channels of varying size, through which solute diffusion occurs. The diffusivities of all tested solutes could be attributed primarily to hydrodynamic and entropic exclusion and only slightly to partitioning and adsorption in the case of low molecular weight molecules [28].

Medical Applications

The biocompatible nature of cellulose-based materials, such as oxidized cellulose, regenerated cellulose hydrogels, sponge cellulose and bacterial cellulose, has allowed comprehensive research targeted at medical applications [31-35]. Representative examples BC-based scaffolds for tissue engineering include vascular grafts, cartilage, neural regeneration and wound dressings.

The interaction between cells and BC has been investigated by several research groups. In 1993, BC was described as a substrate for mammalian cell culture by Watanabe and colleagues [9]. Adhesion to BC was observed using anchorage-dependent cell lines (L929 mouse fibroblasts, Detroit 551, HEL, mouse 3T3 Swiss, SV40/Balb 3T3, CHO, Human J-111 and Human epidermal Keratinocytes). Modification of the BC surface, to improve the interaction with cells, involved the introduction of electrical charge and adhesive proteins, such as collagen type I, collagen type IV, fibrin, fibronectin or laminin [9]. Andrade *et al*. improved the adhesion of fibroblasts on BC pellicles modified using four recombinant proteins containing cellulose-binding module and an adhesion peptide [36].

The interaction of BC films with human transformed skin keratinocytes and human normal skin fibroblasts was evaluated [27]. The results demonstrated that BC supports the proliferation of both cell types, with no signs of toxicity; the keratinocytes exhibited normal cell proliferation, spreading and also maintained the normal phenotype, while for the

fibroblast culture the pattern of cell distribution and stability on BC film was poorer. Moreover, the migration of keratinocytes on a BC film was comparable to that of a polystyrene plate. Pértile and colleagues, in 2007, found a similar behavior when studying the interaction between BC pellicles and skin fibroblasts. Figure 3 shows a detail of the BC network with fibroblast cells adhered on BC membranes [37].

In an *in vivo* biocompatibility study, BC was subcutaneously implanted in mice, for a period of up to 12 weeks [3]. BC was shown to integrate well into the host tissue, with cells infiltrating the BC network and no signs of chronic inflammatory reaction or capsule formation. The formation of new blood vessels around and inside the implants was also observed, evidencing the good biocompatibility of the biomaterial. Figure 4 presents unpublished results obtained by the authors of this chapter. Hystological analysis of BC subcutaneous implants reveals that cells are able to migrate through the cellulose network.

BC and cartilage repair - Svensson and colleagues utilized unmodified and chemically treated BC as a substrate for primary bovine and human chondrocytes culture, aiming at constructing a cartilage tissue with native mechanical properties [10]. Phophorylation and sulfation of the matrices were carried out, in order to add surface charges mimicking the glucosaminoglycans of cartilage *in vivo*. The authors found that BC scaffolds support the growth of chondrocytes, allowing cell migration and ingrowth. Furthermore, these cells did not differentiate into fibroblasts.. On the other hand, human chondrocytes cultivated on unmodified BC express collagen I and collagen II B.

BC membranes were also tested as a new material for the reconstruction and rehabilitation of the nasal framework in rabbits. Good integration of the BC with the nasal septum submucosal space of the animals and some absorption of BC blocks was observed [38]. According to a study by Bodin and colleagues (2007), the BC mechanical properties outcome those of collagen. While BC and pig's meniscus have similar Young modulus, at higher compression strain, the pig's meniscus is stronger than BC [39].

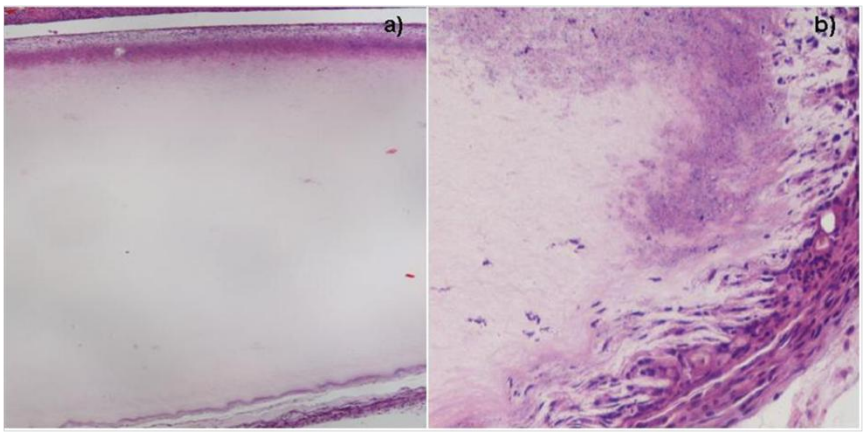

Figure 4. Bacterial cellulose implants 12 weeks post-implantation in mice. (A) The compact (top) and porous side (bottom) of BC implants (40x). (B) Interface area between host tissue and BC implants porous side (200x). Hematoxilin-eosin staining.

BC as vascular grafts - In 2006, Backdhal and colleagues evaluated BC as a novel biomaterial for the tissue engineered blood vessels [25]. The BC was compared to similar

structures of porcine carotid artery (PCA) and expanded-polytetraflourethylene (ePTFE). The mechanical properties of the BC rings were comparable to those of PCA, although PCA significantly exceeded BC in both stress and strain at break and Young's modulus. The authors also studied the interaction between BC and smooth muscle cells (SMCs), the compact and porous side of the BC pellicle being separately analyzed. The results showed that, although the attachment and proliferation of SMCs cultured on the compact and porous sides were similar, differences in the morphology were observed. Furthermore, a maximum ingrowth distance on the porous side of 20μm and 40μm was observed after 1 and 2 weeks, respectively, the more compact side allowing an ingrowth of up to 1-5μm depth. The results revealed that the cells could push the nanofibrils aside, while migrating into the cellulose nanofibril network. In 2008, the same research group developed a novel method to prepare three dimensional nanofibril network tubes from BC with controlled microporosity, by placing paraffin wax and starch particles of various sizes in a growing culture of *G. xylinus*[40]. Smooth muscle cells (SMC) migrate into this more porous cellulose to a greater depth than in the native BC pellicle. The SMCs produced collagen fibers both in the surface cell layer and further into the scaffolds made with paraffin. However, a mechanical evaluation of the SMC-seeded scaffold was not performed.

The effectiveness of angioplasty using conventional stents was compared to bacterial cellulose coated stents, in a rabbit model, by Negrão *et al*. [41]. The authors showed that BC coated stents do not present adverse events in the angioplastic procedures. Indeed, BC accelerated re-endotelialization of the area covered by the stent, acting as a barrier to the migration of muscle cells, thus representing a promising strategy for the prevention and treatment of restenosis in endovascular procedures.

Bodin *et al.* analyzed the growth of endothelial cells (ECs) in the lumen of BC tubes obtained by culturing the bacteria under different concentrations of oxygen [26]. All tubes had a denser inner side and a more porous outer side. The cross section observation revealed layered tube walls, the number of layers and the yield of cellulose increasing with the oxygen pressure. The cells were able to growth in the tubes, forming a confluent layer after 7 days. The same group developed a novel method to graft the RGD cell adhesion peptide on the cellulose, to enhance cell adhesion [26]. The cellulose was modified with xyloglucan and xyloglucan bearing the GRGDS peptide. The results revealed that the nanocellulose material was homogeneously modified; also, cell adhesion studies confirmed a faster adhesion of endothelial cells on the xyloglucan-GRGDS-modified cellulose.

Klemm *et al*. 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 [5]. In this study, four weeks after implantation, the carotid artery-BASIC complex was wrapped up with connective tissue and the BC tube was completely incorporated in the body without any rejection reaction. Putra *et al.* described a simple technique that allows to obtaining a tubular – BC gel with desired length, inner diameter and thickness, along with an oriented fibril structure [42]. This technique requires a shorter cultivation time, as compared to the methodology described by Klemm *et al.* [5]. Our research group also produces BC tubes through a culture system schematized in Figure 5.

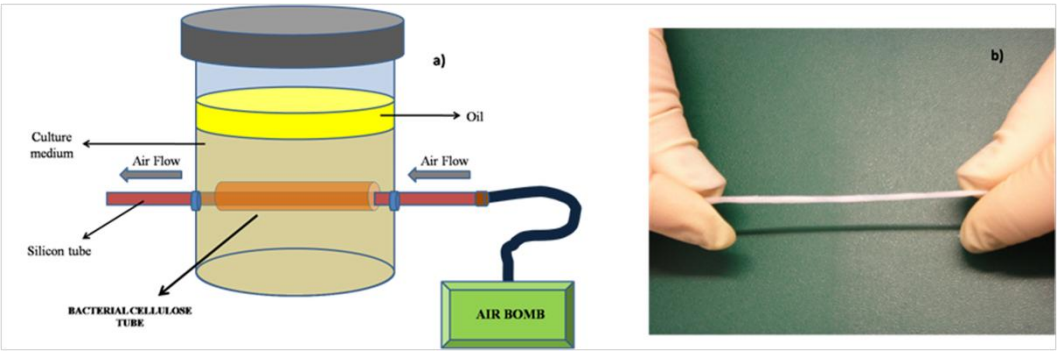

Figure 5. Bacterial cellulose, as produced by *G. xylinus* (ATCC 53582), is synthesized around the silicon tube, when an air flow is injected through the tube. a) Schematic picture of the cultivation system; b) Bacterial cellulose tube.

Over a period of 12 weeks, implanted BC grafts in the carotid artery of pigs showed good *in situ* tissue regeneration without signs of thrombosis, inflammation or fibrotic capsule formation around the implants. The luminal wall of the newly formed tissue showed complete endothelialisation, with a confluent endothelial layer [43].

BC in wound dressing - The Biofill® (BioFill Produtos Biotecnológicos, Curitiba, PR, Brazil) membranes were the first bacterial cellulose membranes to be used in clinical trials [44-47]. Pippi and Sampaio, in 1990, tested the Biofill performance in corneal healing in rabbits submitted to a lamellar keratoplasty [48]. They observed improved corneal healing when the membranes remained attached for longer periods. Wouk and colleagues compared different skin healing promoters, in swine, reporting that BioFill® membranes were more effective than other occlusive dressings tested [49]. The several criteria analysed - healing quality and adhesion to the wound – revealed a good performance of BioFill®. Also the histopathology showed that the deposition of fibrin in the wound occur early when using BioFill®. These membranes do not require daily exchange, normally mandatory with other wound dressings. In 2007, Osman and collaborators published a study comparing the healing of the lateral wall of the nose of rabbits after turbinectomy, using Bionext® (Bionext Produtos Biotecnológicos Ltda, São Paulo, SP, Brazil) membranes as temporary dressing, but they found no difference in hemorrhagea and inflammation between animals with or without the membranes [50].

Mello *et al.*, in 1997 evaluated the action of bacterial cellulose, when implanted as a dural substitute exposed to intact and damaged brain of dogs, evaluating its antifibrotic effect in extradural covering. The experiment showed low foreign body reactions, a decrease of neodural tissue and BC thickness and the absence of cortical adhesion, even in the presence of damage, qualifying this material as a dural substitute [51].

Alvarez *et al.* in 2004 compared biocellulose (BWD, XCell®, Xylos Corporation, Langhorne, Pennsylvania) and a standard nonadherent primary wound dressing, in patients with chronic venous insufficiency and lower-leg ulceration [52]. The authors found that BC dressings create a protective, hypoxic, moist environment and were significantly more effective than standard care for reduction in the amount of nonviable tissue. Furthermore, the average number of days for wound granulation and wound area reduction decreased by

almost half. The wound pain also decreased in patients with BC treatment, as compared to the standard care.

Portal and colleagues used BC wound dressing Dermafill® (AMD/Ritmed, Tonawanda, NY) to assist the repair of chronic lower extremity ulcers, in humans [53]. The BC dressing allowed a reduction of time for epithelization, shortening the time for wound closure over standard care. Panerari tested BC dressings in the rabbit's laryngotracheal region and assessed subglottic tissue response after scarification, attempting to avoid the exuberant scarring tissue formation, often responsible for failure of tracheal stenosis surgery [54]. However, they did not observe differences in inflammation and scarring between the study and control groups.

BC in dental implants – BC Gengiflex® membranes have been used for periondontal disease treatment, dental implants and guided bone regeneration - alone or in association with osteointegrated implants - proving a good alternative for guided tissue regeneration [55-59]. I*n vitro* analysis conducted with osteoblasts showed that these cells produce a collagenous matrix when in contact with BC membranes [60]. Gengiflex® was used by Novaes and colleagues for the regeneration of Class II furcation lesions, in dogs with naturally occurring periodontal disease, through guided tissue regeneration technique [56]. The group concluded that, following 4 weeks of implantation, these membranes can lead to the regeneration of the lesions by forming a barrier membrane maintaining a space for cell migration and tissue formation. Sonohara and Grechi compared Gengiflex, Teflon and Millipore membranes in guided tissue regeneration, on the rat subcutaneous connective tissue, showing that the most biocompatible materials were Teflon and Millipore membranes, while Gengiflex apparently was a stranger material to the host tissue, as evidenced by the presence of a large number of giants cells in contact with the material and a denser fibrous capsule, in comparison to the other tested materials [61]. Macedo and colleagues compared two types of physical barriers, PTFE and Gengiflex membranes, used for guided tissue regeneration of bone defects in rabbits. The results demonstrated that PTFE was more effective in inducing natural bone tissue regeneration than Gengiflex membranes [62].

BC in nerve regeneration - Klemm *et al*. tested micronerve reconstruction of rat sciatic nerve using BASYC® [5]. The regeneration of the functional nerve, following 10 weeks of surgery, was improved as compared to the uncovered anastomosed nerve. The reappearance of acetylcholine as the transmitter of nerve impulses to the executive organ was observed. In the same animal model, the BASYC® was used as a drug depot of neuroregenerative substances, allowing an earlier return of innervation and the functional recreation of the paralyzed legs, as evaluated by the walking behavior scores. Mello *et al.*, in 2001, used bacterial cellulose sheets to envelop peripheral nerve lesions with loss of neural substance, in dogs, and analyzed the degree of inflammatory reaction and axon realignment in the sciatic nerve [63]. A moderate fibrous reaction caused by the BC sheets implanted in the peripheral nerve, and also realignment and axonal growth through the injury were observed. Brancher and Torres observed rats' facial nerves repair following trans-section [64]. The extremities were approximated with a plain epineural suture stitch and surrounded with BioFill® sheets. The researchers found that the BC sheets improved guidance of the nerve fibers, allowing the concentration of neurotrophic factors, which consequently promotes the nerve regeneration.

Improving the Bacterial Cellulose Properties for Biomedical Applications

Biocompatibility is one of the main requirements for any biomedical material. It can be defined as the ability to remain in contact with living tissue without causing any toxic or allergic side effects, simultaneously performing its function [8]. In this context, BC has been modified to further enhance its' biocompatibility. Depending on the envisaged biomedical application, improved cellulose integration with the host tissue, increased degradation in vivo or modified mechanical properties, to mimic the tissue to be replaced, are required. Chemical surface modifications, incorporation of bioactive molecules, modification of the porosity and crystallinity, design of 3D structures and nanocomposites, are examples of viable methods to make BC an ideal material for reparative tissue engineering.

The attachment of cells to biomedical materials can be improved by using adhesion molecules, present in the extracellular matrix substances, such as fibronectin, vitronectin, or laminin. The amino acid sequence Arg-Gly-Asp (RGD) has long been recognized for its cellular adhesion function. Bodin *et al*. described a novel method to activate the bacterial cellulose surface with the RGD peptide, to enhance cell adhesion [26]. The adsorption of modified xyloglucan (GRGDS-xyloglucan) increased the BC wettability, which might explain the decreased or even negligible amount of adsorbed protein. Modification with xyloglucan (XG) did not alter the BC morphology. The water contact angle was lower on BC modified with XG (29 ± 4.8) and XG-GRGDS (32 ± 5.8), when compared to unmodified material (44 ± 5.3). Whitney *et al*. also used xyloglucan to modify BC, in this case incorporating the polyssaccharide in the *G. xylinus* culture medium. These authors verified that XG binds to cellulose, altering its cristallinitty [65]. The RGD was also used by Andrade *et al*. who produced recombinant proteins containing adhesion peptides (RGD or GRGDY) fused to a carbohydratebinding module with affinity to cellulose (CBM3) [36]. The BC was treated with each of the recombinant proteins and tested in fibroblast cell cultures. The results showed that only the proteins with the RGD sequence - adsorbed to BC membranes - significantly improved fibroblasts adhesion, as compared to the unmodified membrane. The results also demonstrated that the protein containing a single RGD copy had, surprisingly, a stronger effect than the protein containing two copies of RGD.

In order to improve cell adhesion to BC, Watanabe and co-workers developed several methods of chemical modification, aiming the introduction of electrical charge to the BC membrane [9]. In this context, membranes of trimethyl ammonium betahydroxy propyl-BC (TMAHP-BC), diethyl aminoethyl-BC (DEAE-BC), aminoethyl-BC (AE-BC) and carboxymethyl-BC (CM-BC) were produced. Also, the TMAHP-BC was covered with adhesive proteins (collagen type I, collagen type IV, fibrin, fibronectin or laminin). The new bacterial cellulose substrates favored the adhesion of cells, as compared to the unmodified BC.

Phosphorylation and sulfation of BC matrices were explored by Svensson *et al*. as a means to add surface charges, mimicking the glucosaminoglycans of cartilage tissue *in vivo* [10]. The materials were analyzed for mechanical properties, microstructure and cell–material interactions, in order to assess the potential of this matrix as a scaffold for cartilage tissue engineering. The compressive modulus of the phosphorylated samples increased with the reaction time and was higher than the compressive modulus of native BC. This result was probably due to the more compact structure of the 3D network in the phosphorylated BC. An even more compact network structure was found in sulfated BC, which showed an higher

resistance to compressive forces when compared to phosphorylated and native BC. sulfated-BC had significantly lower Young's modulus than the unmodified BC, resulting in a reduction of the mechanical integrity. The lower strength of sulfated-BC may be due to the prevention of hydrogen bonding between the cellulose chains by the covalently bonded sulfate groups, chain scission by acid hydrolysis, or a combination of both.

It is known that BC properties such as the mechanical strength and permeability can be changed by post production (*ex situ*) treatments [66]. As an example, treatment with sodium hydroxide is widely used to clean the cellulose membranes, after fermentation, for biomedical applications. George *et al*. analyzed the effect of various alkali treatment methods for BC cleaning, such as potassium hydroxide, sodium carbonate and potassium carbonate. According to these authors, any of these chemicals is milder than the sodium hydroxide, better preserving the BC integrity, and improving the tensile strength of the membranes [67].

Backdhal *et al.* developed a method to produce a highly porous BC [40]. The authors added paraffin wax and starch particles of various sizes to the growing culture of *G. xylinus*. Bacterial cellulose scaffolds with different porosities and interconnectivity were prepared through this approach. The partially fused paraffin particles were incorporated throughout the scaffold, while starch particles were found only in the outermost area of the resulting scaffold. This methodology allows the modulation of the porosity, thickness and interconnectivity of tubular BC scaffolds, by varying the porogen size and fermentation conditions. In addition, the porogens can be successfully removed from the BC network.

As *G. xylinus* has been reported to move along cellulose rails while secreting BC, Uraki *et al*. attempted to expand the utilization of BC and developed novel functional biomaterials, through the transformation of BC into a honeycomb-patterned material [68]. Fabrication of such patterned BC structure was possible by controlling the bacterial movement using an agarose film scaffold with honeycomb-patterned grooves, in a humid CO_2 atmosphere. The results suggest that the obtained honeycomb-patterned network is a continuous porous film, built up with highly oriented and Iα cellulose microfibrils.

Aiming at the production of a *in vivo* degradable polysaccharide , while exhibiting both chitin- and cellulose-like properties , attempts were made to incorporate *N*-acetylglucosamine (GlcNac) residues into bacterial cellulose. Ogawa characterized the enzymatic susceptibility of BC containing *N*-acetylglucosamine (N-AcGBC) residues for cellulase, lysozyme and chitinase hydrolysis [69]. The results showed that N-AcGBC posses high susceptibility for lysozyme (proportional to the GlcNac content) and cellulase but only slight susceptibility for chitinase. The random distribution of GlcNAc residues on N-AcGBC is responsible for the higher lysozyme reactivity. This approach was also studied by Shirai, who described a *G. xylinus* strain adapted to a medium containing GlcNAc, that was used to prepare a novel cellulosic polysaccharide containing residual GlcNAc [70]. The resulting polysaccharide was lysozyme-susceptible. The aminosugar content in the pellicles was measured after cultivation of the bacteria in the presence of various ammonium salts. Ammonium chloride seems to be the best additive to enhance GlcNAc incorporation, under rotatory and aerobic conditions. The acceleration of GlcNAc incorporation in the presence of ammonium salts seems to be due to the shift of the aminotransferase equilibrium in the presence of a high concentration of ammonium ion. The production of similar polysaccharides was obtained by incubation of *G. xylinus* in a modified HestrinSchramm medium containing lysozyme-susceptible phosphoryl chitin (P-chitin) and D-glucose [71]. Analysis of the culture medium by HPLC showed that the P-chitin is depolymerized to monomeric and oligomeric residues, during the incubation,

and these were utilized as a carbon source by the bacteria. Furthermore, monomeric GlcNAc 6-phosphate was also found to enhance the incorporation of GlcNAc residues into the polysaccharide. Also, Ciechanska obtained a modified bacterial cellulose by adding chitosan to the culture medium during the bacterial growth [73]. By FTIR analysis, Glucosamine and N-acetylglucosamine units were shown to have been incorporated into the cellulose chain, providing a material with good mechanical properties in the wet state, high moisture-keeping properties, release of oligosaccharides under lysozyme action, and bacteriostatic activity.

Lee and colleagues investigated the flexibility of the BC synthesis apparatus of a *G. xylinus* strain in incorporating different monomers present in culture medium [72]. The bacteria incorporated 2-amino-2-deoxy-D-glucose (glucosamine) and 2-acetamido-2-deoxy-D-glucose (*N*-acetylglucosamine), but not 3-*O*-methyl-D-glucose or 2-deoxy- D-glucose into the exopolymer. The average molar percentage of glucosamine and *N*-acetylglucosamine in the exopolymers amounted to about 18%. The authors suggested that the cellulose synthase and other enzymes involved in the cellulose synthesis have broad specificity. Preliminary analysis of the fibers (cellulose and the new copolymers) by environmental scanning electron microscopy suggested similar gross morphology (*e.g.* diameter and surface smoothness).
Kobayashi *et al*. produced a cellulose-chitin hybrid polysaccharide by enzymatic polymerization, using a chitinase and a cellulase from *Trichoderma viride* [74]. The molecular weight values of the cellulose-chitin hybrid polysaccharides reached 4030 and 2840, which correspond to 22 and 16 saccharide units, respectively. These MW are rather low compared with naturally occurring chitin and cellulose. The produced cellulose-chitin hybrid polysaccharide did not exhibit a crystalline structure and was hydrolyzed *in vitro* by lysozyme. Also, Phisalaphong and Jatupaiboon obtained a nanostructured BC-chitosan composite, by supplementing the BC culture medium with low-molecular-weight chitosan [75]. Films with a denser fibril structure, smaller pore diameter and higher surface area than the native BC were obtained; however no significant influence in the crystallinity and anti-microbial activity were observed.

Another type of BC modification was described in 2008 by Berti *et al.* [76]. This group produced membranes of BC-PHA by mixing BC olygomers with polyhydroxyalkanoates produced by *R. eutropha* and *C. violacium*, obtaining membranes with different surface properties and porosities.

BC Nanocomposites used in Medicine - According to Ajayan *et al*., nanocomposites can be described as solid structures with nanometer-scale dimensional repeat distances between the different structural phases [77]. These materials typically consist of two or more inorganic/organic phases in some combinatorial form. At least one of the phases or features must be in the nanosize scale. In general, nanocomposite materials demonstrate new and/or improved mechanical, electrical, optical, electrochemical, catalytic or structural properties.

Polyvinyl alcohol (PVA) is a hydrophilic biocompatible polymer with characteristics suitable for biomedical applications. Combined with BC fibres, it has been used to develop biocompatible nanocomposites [78-80]. The PVA–BC nanocomposite is highly anisotropic and its properties make it comparable to heart valve tissue. According to the authors, PVA–BC nanocomposite with specific composition and processing parameters can be obtained to create a custom-designed biomaterial mimetizing the mechanical properties of the tissue to be replaced.

A composite named CollagenBC was developed by Wiegand, by adding collagen type I to the culture medium of BC fermentation, for the treatment of chronic wounds [81]. This composite induces an *in vitro* reduction of protease activity, interleukin concentration and reactive oxygen species, relevant features to support the healing process in chronic wounds. This composite combines the ability of collagen to alter the milieu parameters in chronic wounds, with the excellent BC physical properties. Following the same approach, Zhou and colleagues used a culture medium containing sodium alginate (NaAlg) for the production of BC, obtaining a cellulose with lower cristallinity and a smaller crystallite size [82]. Also, Phisalophong developed a BC-alginate blend exhibiting improved water absorption capacity and water vapor transmission rate combined with a smaller pore size, although the tensile strength and elongation at break of the film decreased [83].

BC is composed of dense microfibrils forming a mat with relatively small pore sizes, the pure BC lacking a suitable pore structure essential for tissue engineering scaffolds. In contrast, the Hydroxyapatite/BC (Hap/BC) nanocomposite scaffolds combine good mechanical properties with an open pore structure, suitable candidates for tissue engineering applications. With the purpose of evaluating the potential of porous Hap/BC nanocomposite as a bone tissue engineering scaffold, Fang *et al.* performed *in vitro* assays [84, 85, 86] were the proliferation and osteoblastic differentiation of stromal cells derived from human bone marrow (hBMSC) on Hap/BC nanocomposite was investigated. The results showed that the nanocomposites performed better than pure BC, regarding cell adhesion, due to the improved pore sizes and presence of the inorganic component. In addition, the authors demonstrated that the nanocomposites stimulate cell proliferation while enhancing osteoblastic differentiation of hBMSC, without osteogenic reagents. Other authors have also synthesized and characterized BC-hydroxyapatite scaffolds, for bone regeneration [87-89]. Shi and colleagues used an alkaline treatment to optimize the biomimetic mineralization of BC pellicles [35]. Calcium-deficient carbonate hydroxyapatite/BC (CaDHCAp/BC) nanocomposites were synthesized in a 3D network of BC nanofibers. The alkaline treatment improved the mineralization efficiency, making the CaDHCAp/BC a potential biomaterial for bone tissue engineering.

BC is a very attractive material for wound dressings, providing a moist environment for wound regeneration, resulting in a better healing. However, bacterial cellulose itself has no antimicrobial activity to prevent wound infection. To achieve antimicrobial activity, Maneerung *et al.* impregnated BC with silver nanoparticles, by immersing the BC pellicles in a silver nitrate solution [90]. Sodium borohydride was then used to reduce the absorbed silver ion (Ag+), inside the BC network, to metallic silver nanoparticles. The size and size distribution of the nanoparticles were effectively controlled by adjusting the molar ratio of $NaBH_4$:$AgNO_3$. Under optimal conditions, well dispersed and regular spherical silver nanoparticles were obtained. The freeze-dried silver nanoparticle-impregnated bacterial cellulose exhibited a strong antimicrobial activity against *Escherichia coli* (Gram-negative) and *Staphylococcus aureus* (Gram-positive), bacteria commonly found on contaminated wounds.

Another composite material was recently reported by Charpentier and colleagues: polyester modified with UV/ozone and plasma treatments, bearing improved hydrophilic character, was coated with BC to produce a new hybrid material that presents potential use in vascular prosthetic devices [91].

Haigler *et al.* added carboxymethylated cellulose (CMC) to the *G. xylinus* culture medium and analyzed the properties of the altered BC produced [92]. The results revealed that an alteration of the ribbon assembly occurs in the presence of CMC, often inducing synthesis of separate, intertwining bundles of microfibrils. Similarly, Tajima incorporated water-soluble polymers such as CMC and methyl cellulose in BC, by incubating *G. xylinus* in a medium containing these polymers [93]. Increased BC production and composites with controllable degradability and mechanical strength were obtained. Also Sakairi *et al.* and Chen *et al.* produced CMC-BC composites by adding CMC to the culture medium [94]. The obtained material had ion exchange ability, with enhanced specific adsorption affinity for lead and uranyl ions, as compared to the original CMC and BC. Using the same approach, Whitney and colleagues [95] added mannan-based polysaccharides to the culture medium, and observed the formation of networks with distinct architecture and modification of other molecular features, such as reduction of crystallinity. A range of different cellulose-associated networks could be formed, depending of the levels of glucomannan and galactomannans added.

Many other studies enlarge the repertoire of different bacterial cellulose composites with potential biomedical application. Different processes were developed to produce BC nanocomposites filled with silica particles, yielding improved elastic modulus and strength, as compared to native BC [96]. Serafica *et al.* produced BC in a rotating disk bioreactor. Several kinds of solid particles (silica gel, iron, aluminum, glass beads, etc) were added to the medium, as the gel forms, being trapped to form new classes of composite materials [97]. Other works report the production of BC – nanocomposites, for example BC/starch [98], BC reinforced with cellulose acetate butyrate [99], BC/glucoronoxylan blends [100], BC/poly dimethylacrilamide and BC/gelatin [101, 102].

BC in Non-Biomedical Applications

Ever since the isolation of *G. xylinus*, by Brown in 1886, the unique properties revealed by BC have allowed embracing an ever growing field of applications, exceeding the biomedical domain [2].

Food applications - The cellulose mat associated with the production of vinegar, Kobucha tea and Nata de Coco has been used for centuries. Nata, a food product originally from the Philippines, became one of the first commercially available products of microbial cellulose, gaining notable popularity in other Asian countries including Indonesia, Japan and Taiwain. This product is traditionally obtained by the static fermentation of coconut wastewaters in shallow trays. The highest demand in Nata products occurred during the nineties, mostly for consumption as a dessert. One interesting feature of the Nata culture is that a stable cellulose producing strain can be maintained over many generations and hence many years. Also, efficient sucrose-utilizing strains have been selected over the years, allowing the use of relatively low cost substrates [2, 103-106].

The high commercialization potential of Nata has lead to intensive research by several major Japanese companies and national governmental organizations which have delineated interdisciplinary research programs with the major goal to develop efficient mass production techniques. This enterprise was designated by “Biopolymer Research Co., Ltd” and was

supported by the Japan Key Technology Center, a joint organization under the Ministry of International Trade and Industry (MITI) and the Ministry of Post and Telecommunications, together with six private companies: Ajinomoto, Shimazu Construction, Nikki, Mitsubishi Paper, Nikkiso and Nakamori Vinegar [107].

Other researchers have suggested that BC could be widely applicable to processed foods to improve their quality; due to its unique properties, BC could be used as a low-calorie additive, thickener, stabilizer, texture modifier, as pasty condiments [103, 108-110] and as a vegetarian foodstuff [111, 112].

Recently, a bacterially produced cellulose film containing nisin was developed and used in a proof-of-concept study to effectively control *Listeria monocytogenes* and total aerobic bacteria on the surface of vacuum-packaged frankfurters [113], thus opening the way to develop self-assembled BC films as active food packaging materials.

BC in papermaking – The proposal of BC as the ideal candidate to replace plant cellulose has been supported by the wide acknowledgement that forest resources must be preserved, ultimately preventing global warming. The heterogeneous composition of the cell-wall matrix and the high source-to-source variability challenge the development of efficient processes for the large-scale liberation of cellulose fibrils, the existing laboratory scale methods often being laborious and time-consuming. The high purity of BC is an interesting alternative to vegetable sources, as it allows obviating purification steps. Contrarily to vegetable sources, the properties of BC can be easily standardized by the type of strain and culture conditions. Also, the superior properties of BC, such as excellent mechanical properties, high thermal stability [114] further justify the interest in papermaking. Using three culture conditions [115], static tray, shaking flask and a stirring bioreactor, the potential of BC to produce a parchment paper, a high quality paper product, was assessed. Only BC paper from static tray and shaking flask exhibited the characteristics of specialty parchment paper. Mormino and Bungay, in 2003, used a novel fermentation system to produce composites of BC and paper [116]. Gostomski and Bungay, in 2002, described the production of BC using a horizontal bioreactor containing half-submerged, flat, circular disks mounted on a central shaft [117]. These disks are rotated in a trough filled with inoculated medium, allowing the attachment and development of cells and a BC pellicle on the surface of the disks. By the controlled addition of paper slurry (from white copier paper or ordinary newsprint paper), cellulose powders (from Sigmacell) and cotton linters, to the culture media during BC biosynthesis, BC composites could be obtained. 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.

Companies such as Mitsubishi Paper Mills in Japan are also investing a lot of resources in developing microbial cellulose for paper products.

Specialty membranes – Several authors have focused on examining the specific application of BC as ultrafiltration, pervaporation and dialysis membranes [28, 67, 83, 118-124]. Shibazaki *et al.*, in 1993, first observed that BC showed a significantly higher permeation rate and a greater molecular weight cut-off than a commercial regenerated cellulose dialysis membrane [118]. The mechanical strength of BC also allowed the use of

thinner membranes. Takai, in 1994, used BC composites obtained by the addition of polyethylene glycol (PEG), carboxymethyl cellulose (CMC), carboxymethyl chitin, and other cellulose-based polymers to the culture medium [119]. All of the tested composites showed lower flux rates when compared to a native BC film. Phisalaphong, in 2008, prepared BC/Alginate blends by casting the mixture over a Teflon plate and gelifying the alginate with $CaCl_2$ [83]. Despite the poorer mechanical properties of the blend membranes when compared to those of pure BC, the composite exhibited improved water adsorption capacity, water vapor transmission rate and degree of swelling in water. Choi *et al*. in 2004 analyzed the feasibility of using bacterial cellulose as a source for environmentally compatible ion-exchange membranes (IEM), to recover heavy metals from industrial wastewater [120]. For this purpose, BC was modified with cation-exchangeable acrylic acid (AAc), by UV-graft polymerization. The modified membrane showed reasonable mechanical properties, such as tensile strength of 12 MPa and elongation of 6.0%.

Evans *et al.* described the ability of BC to catalyze the precipitation of palladium within its structure, generating a high surface area with catalytic potential, a novel and unrecognized property of bacterial cellulose. These authors suggested that the reducing groups of BC were capable of initiating the precipitation of palladium, gold, and silver from aqueous solution. Also, palladium-BC was capable of catalyzing the generation of hydrogen when incubated with sodium dithionite and generated an electrical current from hydrogen in a membrane electrode assembly containing BC as the polyelectrolyte membrane. Palladium-BC was proposed to have applications in the manufacture of both electrical and electronic devices such as biosensors and biofuel cells. The 3D nanofibrilar network structure of BC is compatible with polyelectrolyte membrane (PEM) technology and fuel cell development [125].

Enzymes and cells immobilization – Following their work on the incorporation of solid particles (such as Sepharose and Sephadex resins) onto BC, Serafica, *et al*. were the first to propose the potential of BC for binding interesting bio-chemicals such as enzymes [97]. However only one publication was found were this potential was explored: Wu and Lia immobilized glucoamylase, an enzyme widely used in the food industry, on bacterial cellulose pellets (of 0.5–1.5 mm), via glutaraldehyde coupling [126]. The immobilization enhanced the enzyme's stability against changes in the pH and temperature, especially in the lower temperature region. The relative activity of the immobilized glucoamylase was still above 77% at pH 2.0, the highest value reported in the literature.

Only very recently Rezaee *et al*. published the first reports on the use of BC as a support material for the immobilization of *Pseudomonas stutzeri*, for biological denitrifcation [127, 128]. Following growth under static conditions, the BC was cut into 5-10 mm sections and used for the physical surface immobilization (adsorption) of *P. stutzeri*. The immobilization of the bacterium in BC increased the nitrate adsorption capacity, decreased the cell leakage from the BC pellets, resulted in higher activity of the immobilized cells and allowed better operational control. In the same line of work, *Saccharomyces cerevisiae* was immobilized onto BC, for wine fermentation [129]. The metabolic activities of the immobilized yeast were much higher than those of the free yeast.

BC as display devices – The use of low-cost manufacturing techniques, such as those used in graphic arts printing processes, allowed the manufacturing of organic light emitting

diodes (OLEDs). 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. Shah and Brown (2005) have first put forward the idea of integrating an electronic dye into the nanostructured BC, by synthesizing BC sheets with thickness of around 100 μm and impregnating them with various solutions-processed dopant conductors, to improve their conductivities. This was followed by embedding in an electrochromic dye yielding a high-resolution dynamic display device. The major advantages of such a device include its high paper-like reflectivity, flexibility, contrast and biodegradability. The device has the potential to be extended to various applications, such as e-book tablets, e-newspapers, dynamic wall papers, rewritable maps and learning tools [130].

In a recent publication [131] BC was successfully used as a substrate in flexible organic optoelectronic devices. To be used as OLED substrate, BC was functionalized with a transparent conductive layer, indium tin oxide (ITO). ITO thin films were deposited onto prepared dried BC membranes, at room temperature, using radio frequency magnetron sputtering. 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. Yano *et al*. in 2005 have demonstrated the potential of transparent composites exhibiting low thermal expansion reinforced with BC nanofibers. Because BC nanofibers are virtually free from light scattering, they can reinforce transparent plastics with less than 8% loss of light transmittance, even at a fiber content as high as 70wt% [132, 133]. Nogi and Yano (2008) successfully engineered a foldable and ultra-low-coefficient of thermal expansion (CTE) transparent BC nanocomposite. This nanocomposite exhibited a high optical transparency. Its foldable properties and high thermal stability were achieved by reinforcing a low-Young's-modulus transparent resin (an acrylic resin with 0.7 mm thickness) with 5% BC (a low-CTE and high-Young's-modulus material), taking advantage of the layered structure of planar BC nanofiber networks [134].

BC in Audio components – The first audio speaker diaphragms using microbial cellulose were developed by the Sony Corporation [24, 30]. 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. The costs at the moment are quite high (the headphones retail for about 4,000 USD a set [107]) but as the production techniques become more advanced these should start to come down and the products become more affordable.

BC nano/composites – The cellulose's high availability, renewability, fibrillar structure, high mechanical strength, ability to be multi-functionalized and self-assembled into well-defined architectures, makes it an ideal material for the construction of bio-based nanomaterials [135, 136]. Tailoring how cellulosic interfaces are constructed at the nanoscale may provide the opportunity to develop new materials and products. Also, the cellulose's chemical characteristics provide it with a rich variety of options for chemistry and engineering for material applications. While there is an extensive body of literature using cellulose from vegetable sources, only recently researchers have been gaining a growing

interest in using BC as a substrate for the development of bio-based composites and nanocomposites. Its' relative chemicall purity, thus obviating the purifications steps and the nanometer range of the fiber, facilitate its exploitation. Along with the above mentioned BC modifications aimed at the biomedical field, researchers have engaged in augmenting BC by in-situ modification with polyethylene oxide [137, 138]. By adding either poly(ethylene oxide) (PEO) or poly(vinyl alcohol) (PVA) to the growth medium of *G. xylinus*, finely dispersed bacterial cellulose (BC)/polymer nanocomposites were produced in a wide range of compositions and morphologies, yielding materials with improved thermal and mechanical properties.

Optically transparent materials have attracted a great deal of attention in a wide range of applications such as display devices, coatings and lenses. A biocompatible polymeric nanocomposite was prepared by adding a bacterial cellulose pellicle into the poly(L-lactic acid) (PLLA) matrix [139]. The PLLA/bacterial cellulose nanocomposite retained the transparency of the pure PLLA film due to the nanofibrillar structure of the bacterial cellulose. By field emission scanning electron microscopy, the morphology of the nanocomposite was observed to contain well-dispersed BC fibrils in PLLA. An increase in the mechanical properties was also observed following BC incorporation as a reinforcement into the PLLA matrix.

Self-assembling materials are designed to organize spontaneously and hierarchically into complex structures in appropriate environments. A bioinspired bottom-up process was developed to produce self-assembled nanocomposites of BC bacteria and native starch. 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 bacterial cellulose (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. 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 allowed preserved 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 [89].

Electrospinning, a simple process driven by the electrical forces on the surface of polymeric fluids, producing polymer filaments using an electrostatic force, was used to allow the incorporation of BC whiskers into nanofibers of PEO, thus yield BC/PEO nanocomposite fibers with a diameter of less than 1 μm. The rod-like BC wiskers, as obtained 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. The incorporation of the cellulose whiskers was efficient in enhancing the mechanical properties of the electrospun fibers [140].

Yano *et al*. (2008) [96] explored two processes to prepare nanocomposites of BC filled with silica particles. In one approach, *G. xylinus* was incubated in the culture medium containing commercial silica sol Snowtex 0 (ST 0, diameter 10–20 nm, pH 2–4) or Snowtex 20 (ST 20, also with the same size, pH 9.5–10.0). The elastic modulus was improved by keeping the amount of silica in the nanocomposites below 4% when ST 20 was used and below 8.7% when ST 0 was used. This process allowed incorporation of 50% silica in BC. Inclusion of higher amounts of silica reduced the modulus at and the strength of the nanocomposites below that of BC. In a parallel approach, BC-silica nanocomposites were

obtained by immersion of the the BC hydrogel in different concentrations of silica sols, allowing silica particles to diffuse into the BC hydrogel and lodge in the spaces between the ribbon-shaped fibrils. While this method also increased the modulus and the strength compared to the native BC matrix, it was difficult to load the BC with more than 10% silica. In both processes, the modulus and the strength of the BC nanocomposites made using ST 0 (pH 2–4) were always higher than for those of the nanocomposites made using ST 20 (pH 9.4–10), because *G. xylinus* are more active and productive in an acidic than an alkaline environment.

While the use of BC whiskers (BC-W) has been focused mostly as model fillers in polymeric matrices, their surface-modification [141, 142] is still much underexploited. 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 targeted properties. Their surface modification could improve their stability and their compatibility with the matrices for the synthesis of novel bio-based nanocomposites, with selective solvent dispersibility, surface-activity, stimuli-responsivity and controlled degradability, in high value-added applications such as electromechanical actuators, new nanocatalytic systems, biosensors, enzyme biofuel cells, nano-conductive materials and supercapacitors.

Also, the applications of piezoelectrics to sensors have witnessed a dramatic acceleration. Some of the highlights include electrostrictive materials for positioners, relaxor-normal ferroelectric single crystals with high electromechanical couplings for medical transducers, PZT (lead zirconate titanate) films for microelectromechanical systems, and multilayer actuators [143]. Based on the piezoelectric properties of cellulose, a lightweight, inexpensive, and biodegradable bending actuator from commercial cellophane was obtained, with potential applications in micro-electromechanical systems, biosensors, and flexible electrical displays. Also, under an electric field, a highly oriented film of cellulose nanofibers could be obtained [144, 145]. The electro-active potential of BC and BC-W has not yet been reported in the literature. At CEB-UM/IBB the characterization of the piezoelectricity of BC from *G. xylinus* (ATCC 53582 and ATCC 10245) is on course.

Large Scale Fermentation of BC

As previously mentioned, one of the first commercially available products of microbial cellulose, "Nata de Coco", is obtained by static culture in shallow trays, under non-sterile conditions, using coconut wastewaters in the fermentation broth. This method is inefficient, labor-intensive and time-consuming. Also, final product variability and cross-contamination are major drawbacks. Even under controlled conditions and using defined culture media, scale-up is impractical as large areas and longer incubation periods would be required [106]. The major exploited alternative fermentation technologies, using specific fermentation media and overproducing mutant strains include agitated and air-lift bioreactors, membrane reactors and horizontal bioreactors. Under agitation and aeration conditions, fibrous suspension, or pellets can be obtained. This, however, limits BC applications, allows cellulose-negative mutants to dominate the population of growing cells (which in turn limits the cellulose yield) and requires high agitation power (due to the viscous character of the suspensions). With

membrane bioreactors, cellulose can be obtained as a thick white pellicle with high yields, but major drawbacks include the high operating costs and the difficulty in removing the cellulose product [18, 146-150].

The rotating biological contactor belongs to a heterogeneous phase type of film bioreactor system, with a thin film of growing microorganism attached to a solid support, a liquid medium and a gas phase for oxygen supply. This bioreactor is mostly used to remove organic compounds in municipal wastewaters [151]. The feasibility of the horizontal film reactor for the production of bacterial cellulose and incorporation of particles has been demonstrated by Bungay and Serafica [97, 116, 152, 153]. The configuration involves a stationary liquid phase and rotating disks along a central shaft, passing through the medium. Following initial growth and fibril production, bacteria adhere and proliferate on the rotating supports, producing an exocellulose film. The constructs are alternatively exposed to a gas and liquid phase, leading to efficient oxygen and nutrient transfer. The high surface area-to-volume ratio, ease of operation, ability to change medium composition during fermentation, easy harvesting of the product, are distinct advantages of this system. Also, a significant increase in production rate was observed, when compared to static culture.

Attempting to reproduce the rotating disk system proposed by Bungay and Serafica, at our research centre (CEB-UM/IBB), disks and drums were used as rotating supports for the *G. xylinus* attachment and BC production (Figure 6, unpublished results).

While functional, the major observed inconvenience was the unwanted growth of BC in the culture media, which affected BC production on the disks/drum. Curiously, similar observations were independently recorded by Hornung *et al*. (2007) [154]. In a series of three papers, these authors [154-156] have confirmed that the rate of production of bacterial cellulose stagnates due to the limitation of substrate supply. More interestingly and new to date, they have shown that this growth limitation is caused by a "wall effect", which hinders the removal of the product (cellulose) from the active cell zone. Briefly, this wall effect is characterized by the attrition between the growing BC gel and the inner wall of the beakers and was shown to be the major factor limiting BC production. These authors were pioneers in adapting the principle of direct substrate feeding, a method commonly used in aeroponic cultures [157], for the production of BC, under the premise that, by changing the direction by which the glucose reaches the living bacteria, BC productivity would increase. For this, an aerosol spray system was used to directly and evenly distribute the substrate onto the living bacteria, on the medium-air interface. While promising results were obtained, unwanted effects include recurrent contaminations and the high capital cost of the aerosol. The complex design of the aerosol system was suggested to be a limitation in the removal of adherent microbial contaminants. On ongoing investigation at our research centre (CEB-UM), a potentially more economic system is being used, based in the same principle of a top feeding system. Here, a simpler yet effective and more economical version for the feeding system and sterilization is being tested. An atomizer, a simple and low-cost piece of equipment, is capable of nebulizing a high volume of dispersed microparticles, at controllable dispersion and flow rates (Figure 7, unpublished results). Preliminary experiments have show that the atomizer is capable of an even distribution of the culture media onto to *G. xylinus*.

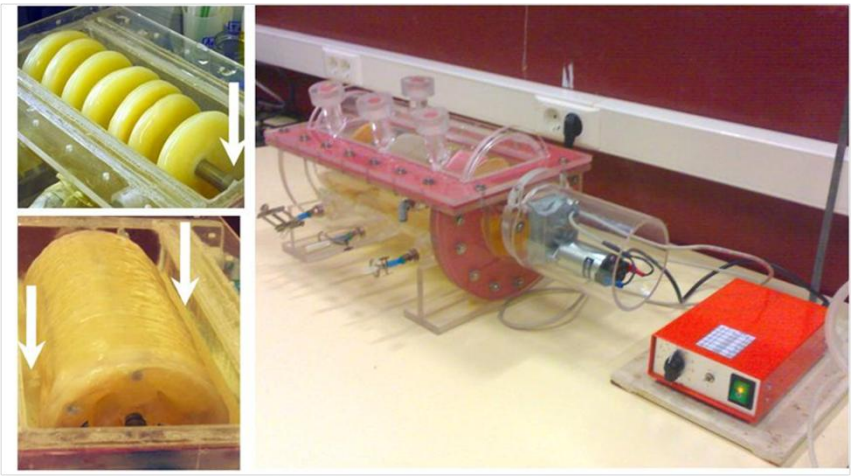

Figure 6. Laboratory-scale horizontal bioreactor developed at CEB-UM/IBB. Figure shows the reactor during operation (right) and the obtained cellulose (left side) using either disks or a cylinder. Along with BC production on the discs and drum, (undesired) accumulation was detected on the surface of the culture media (white arrows).

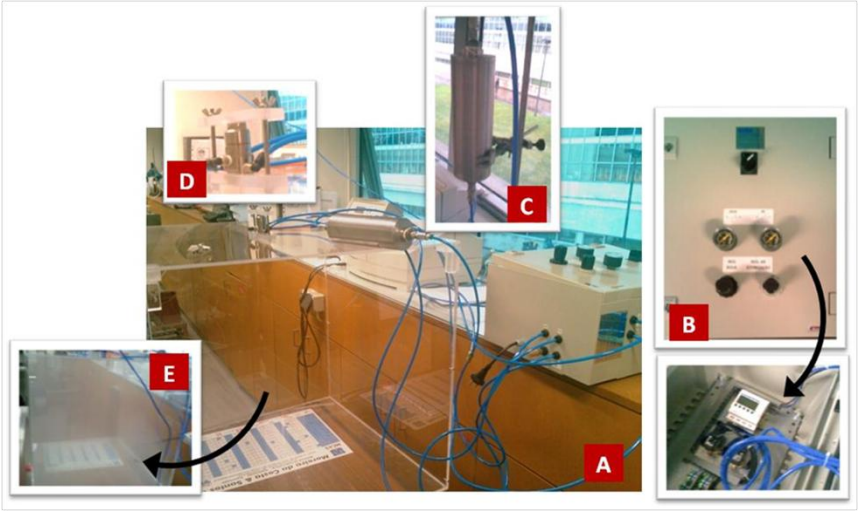

Figure 7. Prototype of the atomizer bioreactor for the surface-culture of BC. Four component unit (A) of the reactor system that includes: the control unit (B), that regulates the flow rate and dispersion rate of the pulverized culture media. This media is retained in a pressurized cylinder (C). Feeding is provided at the top, by pulverization, using the proposed atomizer (D). Figure E shows the atomizer dispersing a thin mist (microparticles) of culture media, filling the atmosphere of the culture box.

From a literature survey, the advantages of most of the existing BC fermentation processes are, at best, suggestive of an economical solution to BC production. A comparative economical analysis between the different technologies has not yet been made. Due to differing fermentation methodologies, strain variability, media culture composition and incubation periods, comparison of the results is difficult. Based on a feasibility analysis by the Central Bank of Indonesia (http://www.bi.go.id/sipuk/en/?id=4&no=52323&idrb=46501) [158] the price of nata de coco rounds up to 460 €/Ton (dry weight). In Portugal, paper pulp

price averages 470 €/Ton (dry weigh; Portucel Soporcel -Portugal, personal communication). For comparison, these values are far below (35%) the calculated average selling price of microcrystalline cellulose by Sigma (150,000€/Ton). Interestingly, the company MB currently sells dried powdered BC at a calculated price of 2,000,000 €/Ton, a value comparable to microcrystalline cellulose. Although the origin of the production is not stated, based on a report from the US Congress (1993) and following currency conversion, the selling price of BC is in average 64,500€/Ton. It is therefore reasonable to expect that BC can in fact be commercialized at competitive prices.

CONCLUSION

Throughout history, humans have greatly relied on the use of materials from biological origin. With the advent of technology, natural polymers came to be tailored to meet specific need as they have come to play a significant role in modern industry. Cellulose is the most abundant natural polymer. While traditionally being extracted from plant tissue, cellulose can also be produced by certain bacterial species by fermentation, yielding a very pure cellulose product with unique properties. Of the several cellulose-producing strains, *G. xylinus* is the only one with the most academic and commercial interest.

In this review, BC was noted to be a unique material with properties allowing applications in virtually every sector of the economy, for example, they can be used as adhesives, absorbents, lubricants, conditioners, drug delivery vehicles and biomedical scaffolds, textiles, high-strength structural materials, packaging and even computational devices. What once started as a scientific curiosity, bacterial cellulose soon became a field on its own. However, most of the studies are today still restricted to the academic level, with few examples of commercial success. Despite its' high promises, a series of limitations must be overpass to enhance the commercial prospect of BC utilization and market expansion. In fact, the economy of scale will need to be improved, as the value-added markets eventually become saturated. Further studies are thus in demand concerning the unraveling of the biochemical pathways leading to cellulose biosynthesis, strain selection, characterization and improvement, especially by way of using the ever-growing tools of genetic manipulation, to better improve the conversion and diversity of substrates, of which, agro-industrial wastes. In parallel to this, an ongoing assessment of the quality of the cellulose product is an important requirement, to meet the various market demands.

ACKNOWLEDGMENTS

The author F.K. Andrade is the recipient of a fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil), and R. A. N. Pértile gratefully acknowledges support by the Programme Alβan, the European Union Programme of High Level Scholarships for Latin America (Scholarship No. E07D401931BR).

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