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Bacterial nanocellulose: biosynthesis and medical application

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ABSTRACT

To meet the steadily growing requirements of humankind for sustainable, functional and smart materials, we contemporarily witness an increasing trend in resorting to emerging technologies like designing bionanocomposites of tailored performance. In this context, bacterial cellulose nanofibers, which are easily accessible by submerse cultivation of diverse, predominately acetogenic, prokaryotic species, constitute a group of auspicious filler- and composite materials which are compatible with various established petrochemical or biological polymers. This opens the door to design bio-inspired, novel composite materials with fascinating physico-chemical characteristics such as low density, tailored gas permeability and high tensile strength. The present article summarizes recent research endeavors in this field, presents examples of nanocellulose-based composites used for "intelligent packaging" and, most of all, it focuses on sophisticated applications in the medicinal field, were rapid degradation of the composite is not desired; here, discussed case studies address smart wound dressing, bone- and cartilage repair, artificial blood vessels, and incorporation and release of antibiotics at controlled kinetics. An outlook demonstrates the perspectives and potential of these novel bionanocomposites on the emerging pharmaceutic and medical market.

KEYWORDS: *Bacterial nanocellulose, bionanocomposites, cellulose biosynthesis, hydrocolloids, medical application, microbial production strains, nanofibers, polyhydroxyalkanoates*

1. INTRODUCTION

Hydrocolloids are colloidal materials characterized by considerable affinity to water which results in the formation of viscous solutions, pseudo-gels, or gels. In dependence on the origin of their synthesis, bio-based hydrocolloids are classified in four categories: Hydrocolloids isolated from animal tissue, from plant materials, produced by microbial fermentation, and, finally, chemically modified plant-derived hydrocolloids are distinguished [1]. As an example for bio-based materials able to form hydrocolloids, cellulose nanofibers occur in a wide variety of organisms, e.g., plants, animals, and some bacteria; they consist of cellulose, the most abundantly available renewable biopolymer on earth, and are accessible at low cost [2,3].

Nanofibers consisting of this environmentally friendly, easily recyclable and renewable polymer are regarded an attractive class of nanomaterials which can be used for manufacturing nanocomposites of low density and high tensile strength at low production cost. As an example, Herrick *et al.* extracted nanofibers from a large quantity of wood pulp by applying high pressure cyclic mechanical treatment. In addition to plant origins, cellulose fibers are also secreted as extracellular products by certain bacteria [4], a promising strategy considering the wealth of well-established techniques for continuous and discontinuous submerse fermentation of bacteria, and the often high product output of such bioprocesses [5,6].

2. BACTERIAL CELLULOSE: BIOSYNTHESIS AND PROERTIES

Cellulose is a linear homopolysaccharide composed of cellobiose, a dimer of 1–4-glycosidically linked β-D-glucopyranose units [7]. An emerging strategy for production of pure cellulose is biosynthesis mediated by acetogenic bacteria such as *Acetobacter* sp., *Acinetobacter* sp. and *Achromobacter* sp., or by the protozoa *Acanthamoeba* sp. The cellulose properties of interest (e.g., molar mass and its distribution), its supramolecular structure as well as synthesis criteria (e.g., kinetics and yield) can be fine-tuned *in statu nascendi* during the bioprocess by carefully selecting the applied substrates, adapting the culture conditions, media composition, and, finally, by choosing the ideal microbial production strain [1,8].

From the microbiological point of view, bacterial cellulose (BC) is assumed to act as a reinforcement component of the bacterial chitosan matrix; this assumption is based on the fact that BC occurs in the form of a pellicle made of highly pure ribbon-shaped cellulose nanofibrils. In contrast to common plant-based lignocellulosic materials, these nanofibrils are "chemically pure", hence, they are devoid of hemicelluloses and lignin. By mimicking nature´s strategy of reinforcing the bacterial chitosan matrix by BC, such nanoribbons may be a suitable filler material for designing chitosan-based composites of technological relevance [9].

Microbial cellulose biosynthesis occurs in the space between the outer and the plasma membrane of the cell by the biocatalytic action of the cellulose synthesizing enzymatic complex starting with uridine diphosphate glucose (UDP-glucose). This complex has a diameter of about 3.5 nm and is associated with pores located at the surface of the bacterial cell. The enzyme cellulose synthase (EC 2.4.1.12) catalyzes the addition of UDP-glucose to the end of growing polymeric chains. Crystallization and polymerization of the elementary fibrils are closely

interconnected. One single microbial cell can convert more than 100 glucose molecules into cellulose per hour [10]. BC is a natural polymer which displays high tensile strength, crystallinity, favorable melting behavior, extremely high degree of polymerization and insolubility in most common solvents. Characteristics of gelatinized BC are similar to the hydrogels produced from synthetic polymers. For instance, BC shows good sorption of liquids and a high water uptake capacity of 98–99% [11]. As a benefit for aspired *in vivo* application, bacterial cellulose (BC) is non-allergenic, and can be safely sterilized by well-established techniques [11]. In the scientific literature, based on the small dimensions of BC-fibers, this material is also known as bacterial nanocellulose (BNC), nanocrystalline cellulose (NCC), cellulose nanocrystals (CNC), or microfibrillated cellulose (MFC) [12]. BC microfibrils feature nanosized diameters of 2–20 nm, which accounts to about 1% of the diameters of plant cellulose fibrils, and fiber lengths in the micrometer range [13].

After the aggregation of elementary fibrils, the resulting microfibrils form two major domains: crystalline and amorphous. The crystalline parts consist of whiskers, also referred to as nanocrystals, nanorods, or rod like cellulose microcrystals [14,15] with typical lengths ranging from 0.5 mm and less than 8–20 nm in diameter, thus resulting in high aspect ratios (high ratio of surface to volume) [14,16]. Microfibrils constitute strings of whiskers, linked by amorphous domains acting as structural defects, and display Young´s moduli similar to those of crystals of native cellulose and a tensile strength of about 10 GPa [17]. The biopolymer degrades at 300 °C and composites produced by incorporation of bacterial cellulose exhibit increased mechanical properties like bending strength and increased Young modulus [18,19]. Especially BC fibers with diameter of less than 10 Å display excellent mechanical properties, e.g., regarding the Young´s modulus and tensile strength. Chemically, the unique network of microfibrils in BC consists of glucan chains bridged by a network of hydrogen bonds. This three-dimensional network leads to a density of 1600 kg m^{-3}, a Young's modulus of 138 GPa, and a tensile strength of at least 2 GPa, which is equal to those of aramid fibers. Dimensions of BC fibrils are in the nanometer range, and they are microscopically similar to collagen fibers [20,21].

3. BACTERIAL CELLULOSE-PRODUCING BACTERIA AND PARTICLUARIETIES OF THEIR CULTIVATION

All representatives of cellulose-forming bacteria excrete this polyglucan as an extracellular product. Similarly, many Gram-negative bacteria and a range of representatives of the Archaea domain secrete extracellular polysaccharides (EPS), predominately heteropolymers acting as protecting biofilms; similar to BC, also these EPS can often be subjected towards promising technological applications such as thickening agents in food technology [22,23]. Apart from production of heteropolymeric EPS, only a few microbes have been revealed to produce the homopolymer cellulose.

Among all bacterial cellulose producers, encompassing species from the genera *Pseudomonas*, *Achromobacter*, *Alcaligenes*, *Aerobacter*, *Azotobacter*, *Agrobacterium*, *Rhizobium*, or *Sarcina*, the strain *Acetobacter xylinum* (also known as *Gluconacetobacter xylinus*) is the most thoroughly characterized organism regarding BC generation [4]. In laboratory praxis, BC is mainly produced at the air–liquid medium interface in HS (Hestrin and Schramm) liquid medium. This medium consists of D-glucose (carbon source), yeast extract (vitamin and nitrogen source), peptone (nitrogen source), citric acid and disodium phosphate (both acting as buffer systems), and distilled water. When oxygen has diminished due to increasing population of respiring bacteria, BC production gets restricted to only those cells having direct access to air bubbles. Other bacteria in the medium are considered resting, but can be reused as inoculum for subsequent cultivation setups [24].

G. xylinus is applied as production strain in industrial scale manufacturing of BNC-based cosmetic masks and high quality wound dressings [20]. The main market for such BNC-derived products, however, is Nata De Coco, a chewy, translucent low-calories dessert, which is popular on the far-east food market. In the field of surgery and regenerative medicine, the production of blood vessels and bone replacements based on BNC have attracted growing interest and resulted in intensive research activities [25,26].

The strain *G. xylinus* E_{25} is described as a highly effective BNC producer, useful for application on industrial scale. BNC biosynthesis by this organism depends on culture conditions (under continuous agitation of the culture broth or under stationary conditions) and presence of ethanol in culture medium, which acts as an additional energy source and directs the glucose flux exclusively towards BC formation [27]. This finding is analogous to the boosted microbial production of PHA-biopolyesters, when ethanol is added as metabolic stress factor to cultures of microbial production strains like, e.g., *Cupriavidus necator* [28]. For efficient, continuous BC production using *G. xylinus*, Krystynowicz *et al.* developed a new tubular horizontal bioreactor, equipped with rotating discs. This equipment enables to overcome reported shortcoming of BC production using this strain under dynamic cultivation conditions, mainly reduced strain stability [29].

Generally, cellulose production by *Acetobacter* sp. is a rather complicated biochemical process and involves three subsequent stages: (a) the polymerization of glucose residues into linear chains of β-1,4-glucan, (b) the extracellular secretion, and (c) the crystallization of the glucan chains into hierarchically structured ribbons [29]. *A. xylinum* (*G. xylinus*) is reported to synthesize two variations of the ribbon- like polymer, and also one amorphous variation which is thermodynamically more stable in comparison to its crystalline counterparts [30].

4. APPLICATIONS OF BACTERIAL CELLULOSE

BC is a nanomaterial applicable for biomedical purposes due to its high purity, degree of polymerization (up to 8000 monomeric units per polymeric molecule), hydrophilicity, biocompatibility, and crystallinity (of 70 to 80%), its high water content of up to 99%, as well as due to its mechanical properties, ultra-fine network, and *in situ* melting ability [8,11]. BC can be used for wound healing, skin engineering (as scaffold after being inoculated by epithelial cells), vascular grafts, as artificial blood vessels (with an inner diameter of about 1 mm), as a potential meniscus implant, or as skin substitute in treating extensive burns [31,32].

The nanostructured network and morphological similarities with collagen make BC very attractive for cell migration and cell immobilization. These important features and uses of BC for practical application are quite different from wood-derived cellulose. As mentioned above, another interesting application of BC is its usage as a calorie-free dessert called Nata de Coco, a common Asian food which gels through the addition of BC produced by *G. xylinum* [8]. BC also can be applied as a collagen-mimicking component in scaffolds due to its beneficial osteoconductive properties [33,34]. Recent research on production of a novel class of bionanocomposites by a combination of hydroxyapatite and BC [35,36] demonstrates its applicability in tissue engineering and bone regeneration [37-39]. Further, the production of BC in the presence of methyl cellulose (MC) and carboxymethyl cellulose (CMC) leads to increased water adsorption capacity of BC, allowing the production of "never dried and reswollen" BC [40].

Additionally, BNC hydrogels may be successfully introduced for *in vivo* cartilage repair by matrix-associated chondrocyte transplantation; currently there is no indication for any *in vivo* biodegradation of BNC in long-term vessel replacement grace to the lack of cellulases in the human metabolism; this qualifies BNC as a sustainable scaffold, supporting cell ingrowth and the subsequent cartilage remodeling in the joint [41].

4.1. Packaging Materials.

In the field of food packaging, microbial biopolyesters like polyhydroxyalkanoates (PHA) can also be processed together with bacterial cellulose nanowhiskers to generate functional bionanocomposites with tailored gas barrier properties (reviewed by [42]). In fact, PHA can be synthesized chemically or, in most cases, by microorganisms and finds wider application, e.g., in development of novel food packaging [43] or in the biomedical and pharmaceutical industry [44,45]. PHA-related research is focused on reduction of production cost by strain improvement and utilization of inexpensive substrates [46-49], design of kinetic models [50], bioreactors and experiments [51,52], as well as on developing new sustainable recovery methods [53,54].

Mechanistically, cellulose nanofibers incorporated into the PHA matrix block the passage for the gas molecules to be transported, similar to effects described for inorganic nanoparticles like clay [55]. Practically, the nanocellulose fibers get incorporated into the PHA matrix using a simple solution casting technique as described by Martínez-Sanz *et al.* These authors demonstrated the high dependence of the gas barrier properties (O_2, CO_2) on the relative humidity, and emphasized that the cellulose nanofiller loading should not exceed a maximum level in order to allow optimum barrier performance [56].

4.2. Veterinary Medicine.

Biosynthetic membranes based on cellulose are biocompatible and applicable in cases of skin tissue loss and burns; mechanistically, they provide selective permeability, and prevent contamination by pathogenic microorganisms. Such membranes have already been used *in vivo* in dog's trochleoplasty; it was shown that they do not interfere with biomechanics and are useful even for intra-articular applications [57-59].

4.3. Dental Medicine.

The nanocellulose products Gengiflex™ and Gore-Tex™ have been used to regenerate dental tissues in humans and support periodontal tissue recovery [57].

5. CELLULOSE-BASED NANOREINFORCEMENTS

Bionanocomposites are usually defined as a combination of two or more materials or phases in which one of the phases has at least one dimension in the nanometer range (1–100 nm) [60-63]. The terminology typically refers to a matrix for the more concentrated component that enables a phase continuum, while reinforcements are components that induce an enhanced performance (e.g., mechanical, thermal, permeability-related, etc.) of the composite. Matrices may be biodegradable polymers (e.g., chemical modified cellulose systems or biopolymers like PHA), ideally derived from renewable resources (e.g., plants) [8,64].

BC used in paper form, so called "nanopapers", possesses high optical transparency, intriguing mechanical properties, low thermal expansion coefficients, and good gas barrier properties; based on its nanoscale pores, these materials can also be used for separation applications [12].

Microfibrils cellulose chains (or nanofibers) in plants or animals are bundles of molecules stabilized through hydrogen bonds [63]. Dependent on the fiber dimensions, two types of nanoreinforcements can be produced from cellulose microfibrils and –whiskers [14], as outlined in the next paragraphs.

5.1. Factors Affecting Performance of Cellulose Nanoreinforcements.

Technically, the production of MFC by fibrillation of cellulose fibers into nanoscale elements requires intensive mechanical treatment such as refining and high-pressure homogenization, cryo-crushing, grinding and pre-treatments e.g., alkaline, oxidation, enzymatic pretreatment [64]. The characteristics of nanocomposites containing cellulose fibers depends on the fibers´ dimensions and aspect ratio, as well as on mechanical and geometric percolation effects [65,66]. Aspect ratios depend also on the origin of the applied cellulose and synthesis conditions of whiskers [14]. Microcrystalline cellulose with diameters on the nanoscale as nanoreinforcement had a higher impact on tensile strength of hydroxyl propyl methyl cellulose (HPMC) in comparison to a micron-sized MCC as filler material.

Also the negative influence of nanoscale MCC on elongation of composite films was less pronounced than its submicron-sized counterparts. Further, composites formed by using whiskers with higher aspect ratio display better tensile properties and higher transparency [67].

5.2. Antimicrobial Activity of Bacterial Nanocellulose.

Although BNC has no intrinsic antimicrobial activity by itself, its complexes with other reagents like silver [68], silver sulfadiazine [69], benzalkonium chloride [70], or montmorillonite [71] can furnish it with antimicrobial properties. Functionalizing BNC with the antiseptic agent polyhexanide (Suprasorb1 X + PHMB) has reached the market as antiseptic wound dressing which is especially effective in the case of critically colonized, severely infected wounds, both superficial or deep [72].

In another work, a complex of BNC with the antiseptic drug octenidine was prepared; kinetics for loading and release of the drug as well as biocompatibility, antimicrobial activity and mechanical characteristics of the complex were investigated in details. It turned out that thickness, ratio of the surface area to volume and water content were pivotal variables significantly influencing the controlled drug release. After incorporation of octenidine into the BNC network, no significant difference was observed in tensile strength and compression. Drug-loaded BNC demonstrated high biocompatibility in human keratinocytes and displayed the desired antimicrobial activity against the pathogenic organism *Staphylococcus aureus* [73].

5.3 Application of Bacterial Nanocellulose for Polyelectrolytic Fuel Cells

In another study, novel proton exchange membranes were produced by incorporation of BNC pulp into the sulfonated tetrafluoroethylene-based fluoropolymer-copolymer Nafion^{TM}. The results showed enhanced Young's modulus, water uptake, swelling and thermal stability as well as reinforced polymeric structure. BNC/Nafion membranes were developed as a promising candidate for a new class of polymer electrolyte membrane fuel cells (PEMFC) and direct methanol fuel cells (DMFC) which are of increasing technological relevance (reviewed by [74]). During the fabrication process of BNC/Nafion membranes, the annealing step is considered to be critical [75].

6. CONCLUSIONS

Since native bacterial nanocellulose can neither be biodegraded in nor excreted from the human body due to the absence of cellulases, applications such as cartilage replacement, temporary skin, wound dressings, connective-tissue replacements as well as scaffolds for tissue engineering, and artificial blood vessels have become of high interest during the last years. Until today, only one BNC-based product containing polyhexanide has reached the market entrance as antiseptic wound dressing; due to the highly dynamic research activities in this field, more products containing active drugs are expected to follow in the not too distant future. Incorporation of antiseptic agents in BNC may pave the way for developing a convenient ready-to-use system for wound treatment with both advanced healing- and superior material properties, combined with an efficient infection control and minimized unwanted side effects. In this context, drug-loaded BNC might enable a controlled release profile comparable to already marketed products, which are stable for several months without losing their desired biological and physicochemical properties. Upscaled research on the pilot scale is needed to identify and optimize parameters influencing the controlled and sustained release of active compounds from the polymeric matrix. Further, *in vivo* research should be carried out on a broader spectrum of infectious microorganisms and different types of wounds.

Due to the unique characteristics of BNC such as small size and heterogeneity of the pores, BNC-hydrogels inhibit the ingrowth of cells and hamper their application as tissue-engineered implant materials. To overcome this limitation, application of placeholders during BNC biosynthesis or post-processing steps such as (touch-free) laser perforation can be recommended. BNC hydrogels may be successfully introduced for *in vivo* cartilage repair by matrix-associated chondrocyte transplantation. The current state of knowledge suggests that this approach is definitely promising: there is currently no indication for any *in vivo* biodegradability of BNC in long-term vessel replacement, qualifying BNC as a sustainable scaffold supporting cell ingrowth and subsequent cartilage remodeling in the joint.

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