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Correlation between type of alkali rinsing, cytotoxicity of bio-nanocellulose and presence of metabolites within cellulose membranes

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ABSTRACT

The study aimed at evaluation of various types of alkali rinsing with regard to their efficacy in terms of removal, not only of bacteria but also bacterial metabolites, from cellulose matrices formed by three *Komagataeibacter xylinus* strains. Moreover, we tested the type of alkali rinsing on membrane cytotoxicity in vitro in fibroblast and osteoblast cells and we compared matrices' ability to induce oxidative stress in macrophages. We identified 11 metabolites of bacterial origin that remained in cellulose after rinsing. Moreover, our results indicated that the type of alkali rinsing should be adjusted to specific *K. xylinus* strains that are used as cellulose producers to obtain safe biomaterials in the context of low cytotoxicity and macrophage induction. The findings have translational importance and may be of direct significance to cellulose dressing manufacturers.

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1. Introduction

Bacterial cellulose (BC) is an exopolysaccharide produced by various species of bacteria; the most effective producers of this polymer are *Komagataeibacter xylinum*, *K. hansenii, K. medellensis* and *K. pasteurianus* (Castro et al., 2012; El-Saied, Basta, & Gobran, 2004; Jung, Park, & Chang, 2005; Yoshino, Asakura, & Toda, 1996). Although the molecular formula of BC $(C_6H_{10}O_5)_n$ is the same as that of plant cellulose, physical and chemical features differ (Tsouko et al., 2015). BC displays higher purity, and exhibits a higher degree of polymerization and crystallinity index as compared to plant cellulose. Fibrils of BC are about 100 times thinner than plant cellulose fibrils (Chawla, Bajaj, Survase, & Singhal, 2009), and also have higher tensile strength, water holding capacity, moldability, and porosity (Czaja, Romanovicz, & Brown, 2004). The organization and hydrogen bonding of a tridimensional network of nano- and micro-fibrils confers unique physiochemical properties to BC, such as high Young's modulus and mechanical strength and also pellicle formation. Further, BC is biocompatible and is thus becoming a promising biopolymer for several biomedical applications such as wound dressings and membranes, artificial skin, and scaffolds for tissue engineering and soft tissue replacement. Technological

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applications of BC exist and include optical transparent nanocomposites, electronic paper, and fuel cell membranes (Evans, O'Neill, Malyvanh, Lee, & Woodward, 2003; Wei, Rodriguez, Renneckar, & Vikesland, 2014; Yano et al., 2005). BC is also used in the food industry as a source of dietary fiber for production of desserts, fruit cocktails and fruit jellies (Jagannath, Raju, & Bawa, 2010).

BC also exhibits superior purity when compared with plant cellulose, so treatment is not required to remove unwanted polymers and contaminants (e.g. lignin, hemicellulose) (Kucińska-Lipka, Gubanska, & Janik, 2015). However, BC obtained after fermentation always contains impurities like bacterial cells and culture media components. The application of BC (especially for medical purposes) requires removal of media residues and bacterial cells containing toxins like lipopolysaccharide (LPS). The most commonly used process of BC purification from bacterial cells is treatment with sodium hydroxide (NaOH) at a concentration of 0.1–1 M and temperature of 80–100 °C for 20–120 min (Krystynowicz et al., 2002; Li, Kim, Lee, Kee, & Oh, 2011; Lin, Lopez-Sanchez, Li, & Li, 2014; Son et al., 2003).

The aim of this process is not only to kill bacteria, but also to remove any other impurities from BC biomaterials such as membranes. Thus, alkali treatment leads to BC biomaterial depyrogenation or drop in LPS concentration to an acceptable level for various applications (Chawla et al., 2009). However, the number of reports investigating residues other than LPS in cellulose biomaterials after NaOH treatment is extremely scant. Also, there are virtually no reports investigating the relationship between different modes of alkali treatment and cytotoxicity of BC biomaterial.

Therefore, the aim of this line of investigation was to compare different modes of NaOH treatment in regards to the level of cytotoxicity displayed by BC membranes. Another aim was to investigate, by means of modern analytical techniques, the presence and composition of molecules other than LPS in BC after purification.

2. Materials and methods

2.1. Production of bacterial cellulose

For the production of cellulose *K. xylinus* DSM 5602 (Deutsche Sammlung von Mikroorganismen und Zellkulturen – German Collection of Microorganisms and Cell Cultures), *K. xylinus* DSM 46602 and *K. xylinus* DSM 46604 were used. The strains were cultivated in stationary conditions for 7 days at 28 °C in a 24-well culture plates (Becton Dickinson and Company, USA) using a Hestrin-Schramm (H-S) medium. The medium was composed of glucose (2 w/v%; POCH, Poland), yeast extract (0.5 w/v%; Graso, Poland), bacto-pepton (0.5 w/v%; Graso), citric acid (0.115 w/v%; POCH), Na_2HPO_4 (0.27 w/v%; POCH), $MgSO_4 \cdot 7H_2O$ (0.05 w/v%; POCH), bacteriological agar (2% w/v; Graso) and ethanol (1 v/v%; POCH).

2.2. The preparation of cellulose samples

BC pellicles were harvested from microbiological media and rinsed with water. Next, to remove bacterial cells and media components the BC samples were purified using 3 different concentrations of NaOH (POCH) solution (0.1 M, 1 M, 2 M) at 90 °C for 30 min. For one group of BC samples, the purification procedure using a particular NaOH concentration was repeated two times (2 × 0.1 M, 2 × 1 M, 2 × 2 M) and for another group, four times (4 × 0.1 M, 4 × 1 M, 4 × 2 M). After purification, BC pellicles were immersed in distilled water and incubated with shaking, while pH value was measured every 3 h. When a drop in the pH value of water was detected (as compared to the initial pH of water used for the purification) the water was changed and the BC samples were incubated for another 3 h. The washing procedure was continued until there was no change in pH (approximately 48 h). As a control, NaOH-untreated BC pellicles were used. The control membranes were produced by washing with distilled water only for 30 min.

2.3. SEM and EDX assessment

The membranes were fixed using 3% glutaraldehyde (POCH) for 15 min at room temperature. Then, the samples were rinsed twice with phosphate buffer (PBS; Sigma Aldrich, Germany) to remove the fixative. The next step was dehydration in increasing concentrations of ethanol (25%, 60%, 95%, and 100%; POCH) for 5 min in each solution. After rinsing off the ethanol, the samples were dried. Then, the samples were covered with gold and palladium (60:40; sputter current, 40 mA; sputter time, 50 s) using a Quorum machine (Quorum International, USA) and examined under a Zeiss EVO MA25 scanning electron microscope (Zeiss, Germany). EDX assessment was performed using Zeiss EVO MA25 built-in device. The EDX results were compared between specific series within samples.

2.4. Neutral red cytotoxicity assay

Neutral Red (NR) cytotoxicity assay was performed in osteoblast (U2-OS) and fibroblast (L929) cell cultures treated with extracts obtained from BC membrane-conditioned medium. The extracts were prepared according ISO 10993 norm: Biological evaluation of medical devices; Part 5: Tests for *in vitro* cytotoxicity; Part 12: Biological evaluation of medical devices, sample preparation and reference materials (ISO 10993-5:2009 and ISO/IEC 17025:2005). Briefly, BC membranes (ca. 2 cm in diameter and ca. 2 mm in thickness) were introduced to the plates' wells filled with appropriate cell culture media without serum (F12 for osteoblasts or MEM for fibroblasts, both media were purchased from Sigma-Aldrich) and incubated for 72 h in 5% CO_2 at 37 °C with shaking at 500 rpm (Schuttler Microplate Shaker, MTS-4, IKA, Germany). After incubation, membranes were extruded from the wells, and the plates were spin-centrifuged. The resulted supernatants (extracts) were next introduced to the cell cultures and incubated for 24 h, 48 h, 72 h in 5% CO2 at 37 °C. After specific time of incubation, medium was removed and 100 μL of NR solution (40 μg/mL; Sigma-Aldrich) was introduced to wells of the plate. Cells were incubated with NR for 2 h at 37 °C. After incubation, the dye was removed, wells were rinsed with PBS (Sigma Aldrich) and left to dry at room temperature. Subsequently, 150 μL of de-stain solution (50% ethanol 96%, 49% deionized water, 1% glacial acetic acid; POCH) was introduced to each well. The plate was vigorously shaken in a microtiter plate shaker for 30 min until NR was extracted from the cells and formed a homogenous solution. Next, the value of NR absorbance was measured spectrometrically using microplate reader (Multiscan GO, Thermo Fisher Scientific, USA) at 540 nm wavelength. The absorbance value of cells not treated with extracts was considered 100% of potential cellular growth (positive control).

2.5. ^1H NMR measurements and data processing

The extracts for ^1H NMR analyses were prepared in the manner described in Section 2.4, the only difference was that PBS buffer (Sigma Aldrich) instead of cell culture media was used for incubation. The NMR spectra were recorded at 300 K using Avance III spectrometer (Bruker, GmBH, Germany) operating at proton frequency of 700.58 MHz. The all one-dimensional ^1H NMR spectra of cellulose membrane extracts were carried out using *noesy1dpr* (in Bruker notation) pulse sequence by suppression of water resonance by presaturation. The following acquisition parameters were applied: spectral width 20 ppm; acquisition time 1.36 s per scan; time domain points for cell extracts 64 K; relaxation delay 3.5 s; number of scans 128. Spectra were processed

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