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In-situ glyoxalization during biosynthesis of bacterial cellulose

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ABSTRACT

A novel method to synthesize highly crosslinked bacterial cellulose (BC) is reported. The glyoxalization is started in-situ, in the culture medium during biosynthesis of cellulose by *Gluconacetobacter medellensis* bacteria. Strong crosslinked networks were formed in the contact areas between extruded cellulose ribbons by reaction with the glyoxal precursors. The crystalline structure of cellulose was preserved while the acidic component of the surface energy was reduced. As a consequence, its predominant acidic character and the relative contribution of the dispersive component increased, endowing the BC network with a higher hydrophobicity. This route for in-situ crosslinking is expected to facilitate other modifications upon biosynthesis of cellulose ribbons by microorganisms and to engineer the strength and surface energy of their networks.

1. Introduction

Bacterial cellulose (BC) is an extracellular biopolymer produced by microorganisms belonging to the *Gluconacetobacter* genus. These bacteria extrude cellulose ribbons that form a three-dimensional network at the air–liquid medium interface. Cellulose chains are assembled into sub-elementary fibrils of 1.5 nm width that further associate to form cellulose nanofibrils (2–4 nm width) and nanofibril bundles or ribbons (20–100 nm width) (Iguchi, Yamanaka, & Budhiono, 2000). BC is highly pure and crystalline and exhibit good water-holding capacity, mechanical strength and degradability (Bielecki, Krystynowicz, Turkiewicz, & Kalinowska, 2005). As a consequence, recent investigations have considered BC as a reinforcing constituent in advanced thermoplastic and thermosetting polymer matrices, resulting in composite materials with unique properties and morphologies (Laborie, 2009).

The hydrophilicity of cellulose is related to the presence of three different OH groups in the repeating units of the polymer which are capable of forming inter- and intra-chain hydrogen bonds. The resultant surface hydrophilicity of cellulose fibrils limits their compatibility with nonpolar matrices. As a result, several functionalization strategies have been attempted, including esterification, polycondensation, etherification, and acetalyzation reactions (Heinze, & Liebert, 2001). Likewise, cellulose can be chemically modified with crosslinking agents to covalently bond the fibrils (Schramm & Rinderer, 2002; Yu, Lee, & Bang, 2008). One of the most commonly used crosslinking agents include formaldehyde-based chemicals but their use has been undermined by reported carcinogenic effects (Schramm, & Rinderer, 2000). Thus, alternative crosslinking agents are needed such as polycarboxylic acid and dialdehydes, which have been used since the late 1980s (Xu, Yang, & Deng, 2001; Lee, & Kim, 2005). Among crosslinking agents, glyoxal has been one of the few readily available non-formaldehyde agents capable of crosslinking cellulose (Schramm & Rinderer, 2002; Welch, 1983; Head, 1958). Glyoxal is the simplest of the dialdehyde group. It is produced by gas-phase oxidation of ethylene glycol, liquid-phase oxidation of acetaldehyde or by lipid autoxidation (Kielhorn, Pohlenz-Michel, Schmidt, & Mangelsdorf, 2004). Moreover, upon release into the environment, glyoxal is rapidly converted enzymatically to glycolate by microorganisms as bacteria and fungi (Kielhorn et al., 2004; Quero et al., 2011).

Reactions with glyoxal have commonly been used to impart durable properties to cellulose-based materials, including textiles and paper (Schramm & Rinderer, 2002; Yu et al., 2008). Surface

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modification of BC can be carried out by impregnation, primarily to reduce their hydrophilic character and prevent delamination of the nanostructure. The effects of surface modification and resulting mechanical properties have been evaluated by Quero et al. who reported a low degree of delamination while preserving the mechanical strength (Quero et al., 2011).

In the present report, we propose a novel method to glyoxalize BC in situ, during synthesis in the culture medium. To this end, the culture medium of *Gluconacetobacter* bacteria was modified with low concentration glyoxal crosslinking agent thereby allowing effective contact with the BC ribbons. The effects of the crosslinking reactions after thermal curing were investigated in terms of changes in morphological, chemical and crystallinity characteristics of BC as determined by scanning and transmission electron microscopy (SEM and TEM), X-ray diffraction (XRD), attenuated total reflection Fourier transform infrared spectroscopy (ATR–FTIR) and CP/MAS ^{13}C nuclear magnetic resonance (NMR). In addition, the changes in the surface properties were evaluated by inverse gas chromatography (IGC) and contact angle (CA) measurement.

2. Materials and methods

2.1. Bacterial cellulose production

The *Gluconacetobacter* strain used in this study was previously isolated from a pellicle of homemade vinegar (Castro et al., 2013). The purified bacterial strain was incubated at 28 °C for 8 days in a static Hestrin–Schramm (HS) culture medium containing 2 w/v% glucose, 0.5 w/v% peptone, 0.5 w/v% yeast extract, 0.27 w/v% Na_2HPO_4, adjusted to pH 3.5 by phosphoric acid (also used as catalyst agent in the crosslinking reaction). Glyoxal was used as crosslinking agent and was added to the culture medium at 0.3 v/v%. It is important to note that glyoxal concentration of 0.3 v/v% is toxic for the microorganism. In this work, an adaptation procedure is applied. The bacteria were exposed to an initial concentration of glyoxal of 0.01 v/v% for 8 days at 28 °C. After this time, a portion of the above medium was inoculated (10 v/v%) in fresh medium with a 0.02 v/v% glyoxal concentration (0.01% increments were made at each change). This procedure was repeated until a concentration of glyoxal of 0.3% was achieved. At each step the phenotypic characteristics of the microorganism and the ability to produce cellulose was verified using the method of Castro et al. (2013).

The BC pellicles collected from the final 0.3% glyoxal containing modified medium (BCM) were dried (40 °C, 48 h) and then cured (120 °C, 5 min) to allow cross-linking and to yield glyoxylated BC (BCG). BCG was washed with distilled water, treated for 14 h in a 5 wt% KOH solution and rinsed until pH 7 to remove the bacterial cells and residual culture medium. A control system consisting of BC with no glyoxal treatment (BC) was produced following the same methodology described for BCG in glyoxal-free culture media.

2.2. Imaging with scanning and transmission electron microscopy (SEM and TEM)

The surface of freeze-dried pellicles was coated with gold/palladium using an ion sputter coater for 5 min and the samples were imaged in a scanning electron microscope (Jeol JSM 5910 LV) using the secondary electron mode at 10 kV.

Cellulose ribbons were extracted from the BC, BCM and BCG pellicle networks by mechanical treatment at 3000 rpm for 30 s. Drops of each suspension were deposited onto glow-discharged carbon-coated electron microscopy grids and negatively stained with 2 wt% uranyl acetate. All samples were observed in a transmission electron microscopy (Philips CM200) operating at 80 kV. The images were recorded on Kodak SO163 films.

2.3. Spectroscopic analyses via FTIR and CP/MAS ^{13}C NMR

Attenuated total reflection Fourier transform infrared spectroscopy (ATR–FTIR) was used for chemical analysis. Before sample analysis, BC and BCG were dried at 40 °C to remove moisture. FTIR spectra were recorded on a Nicolet 6700 spectrophotometer in the 4000–400 cm^{-1} range using ATR. The spectra were recorded with a resolution of 4 cm^{-1} and an accumulation of 64 scans.

^{13}C cross polarization magic angle spinning nuclear magnetic resonance (CP/MAS ^{13}C NMR) was used for analysis of the BC and BCG films on a Bruker AV-400-WB spectrometer with a triple probe channel of 4 mm, with rotors of ZrO and a stopper of Kel-F at room temperature. The speed of rotation was 8 kHz and the pulse sequence employed was cross polarization (CP–MAS) ^1H–^{13}C, using a spectral width of 35 kHz, a contact time of 3 ms and a relaxation time of 4 s with decoupling ^1H. The number of scans was 2048. The chemical shift was established in relative ppm to tetramethylsilane (TMS) as primary reference and the signal of adamantine CH_2 (29.5 ppm) was used as secondary reference.

2.4. X-ray diffraction (XRD)

Dry films of BC and BCG were X-rayed using a Panalytical X'Pert Pro MPD equipment operating at the Ni-filtered Cu$K\alpha_1$ radiation wavelength (λ = 0.15406 nm), generated at a voltage of 45 kV and a filament emission of 40 mA. Data were collected in reflection mode in the 10–30° 2θ-range with a step of 0.013°. The scans proceeded at 56.58 s per step. The peaks were deconvoluted using Pearson VII peak functions for *d*-spacings and apparent crystal size (ACS) determinations. The *d*-spacings between the crystal planes were determined using Bragg's law:

$$d = \frac{\lambda}{2\sin\theta} \quad (1)$$

where θ is the angle between the plane and the diffracted or incident beam and λ is the X-rays wavelength. The ACS was calculated using Scherrer's formula:

$$\mathrm{ACS} = \frac{(0.9\lambda)}{\mathrm{FWHM}\ \cos\theta} \quad (2)$$

where FWHM is the width of the peak at half the maximum height.

2.5. Surface energy via contact angle (CA) measurement and inverse gas chromatography (IGC)

Six sessile contact angles were measured on BC and BCG films using an optical contact angle meter (KSV instruments LTD) at room temperature after deposition of 5 μL ultra pure water drop on the surface.

IGC measurements were carried out using a commercial inverse gas chromatograph (Surface Measurements Systems, London, UK) equipped with flame ionization (FID) and thermal conductivity (TCD) detectors. The data obtained were analyzed in *i*GC Standard v1.3 and Advanced Analysis Software v1.21 (Cordeiro, Gouveia, Moraes, & Amico, 2011). The BC and BCG samples were packed into standard glass silanized columns (ID: 2 mm, length: 30 cm). The columns with the samples were then conditioned in the IGC overnight at 40 °C followed by 2 h at the specific measurement condition.

An alkane series (*n*-heptane, *n*-octane, *n*-nonane and *n*-decane) was used in the measurement of the dispersive surface interactions at 20 °C. For the acid–base studies, and specific surface free energy, acetonitrile, ethyl acetate, ethanol, acetone and tetrahydrofuran were used at the same conditions than that used for dispersive surface interaction measurements. The isotherms were collected with different concentrations of *n*-octane, ethanol and tetrahydrofuran,